PROTECTIVE EFFECT OF SOME NATURAL PRODUCTS ON GLUCOSE OR METHYLGLYOXAL INDUCED GLYCATION OF SUPEROXIDE DISMUTASE

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

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BY

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UNDER THE SUPERVISION OF

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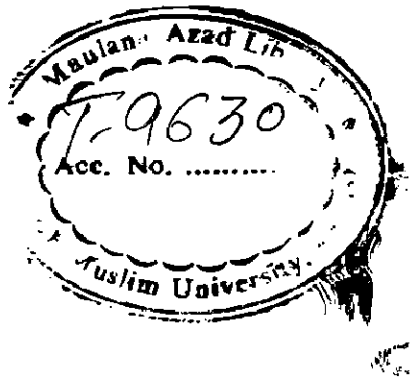

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Dedicated to
My
Parents and my Supervisor

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CONTENTS

ABSTRACT

Diabetes has become the most common metabolic disease worldwide. Glycation plays an important role in various oxidative stress related diseases. Glycation is a non-enzymatic sequence of reactions in which proteins, lipids and nucleic acids react with sugars and lead to the formation of a variety of complex and diverse compounds, collectively described as advanced glycation end products (AGEs). AGEs formation is accompanied by the formation, among others of a number of reactive oxygen species (ROS), α-oxoaldehyde including methylglyoxal (MG), that further react and damage the proteins and other important biological molecules. Among the enzymes inactivated by glycation, superoxide dismutase (SOD) constitutes the first, and possibly, the most important line of antioxidant defense, enabling cells to cope with lethal oxidative environment. Exposure of SOD to glucose results in its deactivation following site-specific and random fragmentation. And exposure of the enzyme to MG has been shown to cause its covalent cross-linking associated with loss of enzymatic activity. The damage caused by oxidative stress is expected to be exacerbated if the antioxidant enzymes themselves are inactivated by glycation.

The identification of antiglycating compounds is attracting considerable interest. Current antidiabetic therapy is based on synthetic drugs that very often have side effects. Alternative medicines and natural therapies have stimulated new interest of research to find for more efficacious agents with lesser side effects. Naturally occurring phytochemicals with anti-diabetic activities are relatively nontoxic, inexpensive and available in an ingestable form. A large number of plants and natural biomolecules have been discussed in literature for their antidiabetic effects. The mechanism is most often not completely understood. A large number of hypoglycaemic compounds have antioxidant properties. Thymoquinone (TQ), *Aloe vera* (*A. vera*), aloin, ellagic acid (EA) and alliin have been reported to have various beneficial pharmacological activities including antidiabetic activity. Therefore, the glycation of SOD by glucose or MG and its protection by the above mentioned natural products has been investigated in this study.

Incubation of SOD with glucose, MG or both at 37°C resulted in a progressive decrease in the activity of the enzyme, a parallel decrease in the amount of protein on

SDS-PAGE gels for glucose incubated SOD and formation of high molecular weight aggregates for MG or both glucose and MG incubated enzyme, and reduced cross-reactivity with anti-SOD antibodies. The structural changes associated with the glycation of the enzyme have been investigated by biophysical techniques. Incubation of SOD with glucose, MG or both at 37°C resulted in progressive hyperchromicity at 280 nm, intrinsic fluorescence quenching at 310 nm, decrease in negative ellipticity at 208 nm, AGEs specific fluorescence enhancement in the wavelength range 400-480 nm, and Thioflavin T (ThT) fluorescence enhancement at 480 nm (fibrillar state enhancement). Therefore glycation by glucose or MG induced both tertiary and secondary structural changes in SOD, and formation of AGEs and fibrils. The changes were more and faster with MG than with glucose since MG is a stronger glycating agent than glucose. TQ, *A. vera*, aloin, EA and alliin offered protection against glucose or MG induced glycation of SOD. The antiglycating activity of these natural products appears to be better for mild glycating agents. The order of decrease of antiglycating potential is: EA/alliin > aloin > TQ. The antiglycating potential of EA and alliin appears to be comparable with that of quercetin which is reported to be a potent natural inhibitor of glycation. Thus, TQ, *A. vera*, aloin, EA and alliin can be used for reducing diabetic complications many of which are due to protein glycation. These natural products have earlier been reported to have antidiabetic effects, and this along with their observed antiglycating effect makes them effective products against diabetes and its complications.

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LIST OF ABBREVIATIONS

AGEs	Advanced glycation endproducts
AG	Aminoguanidine
A. vera	*Aloe vera*
BSA	Bovine serum albumin
CF	Cystic fibrosis
CEL	Carboxyethyl Lysine
3-DG	3-deoxyglucasone
DHAP	Dihydroxyacetone phosphate
EA	Ellagic acid
H_2O_2	Hydrogen peroxide
MG	Methylglyoxal
MOLD	MG lysine dimer
NADH	Nicotinamide adenine dinucleotide (Reduced)
NBT	Nitro blue tetrazolium
$O_2^{\cdot}$	Superoxide anion free radical
PAGE	Polyacrylamide gel electrophoresis
PMS	Phenazine methosulfate
ROS	Reactive oxygen species
SDS	Sodium Dodecyl sulfate
SOD	Superoxide dismutase
ThT	Thioflavin-T
TQ	Thymoquinone

Introduction

1. Introduction

1.1. Superoxide dismutase

Superoxide dismutases (SODs) are a group of metalloenzymes that are found in all kingdoms of life. SODs form the front line of defence against reactive oxygen species (ROS) mediated injury (Kangralkar *et al.*, 2010). These proteins catalyze the dismutation of superoxide anion free radical (O_2^-) into molecular oxygen and hydrogen peroxide (H_2O_2) at a diffusion limited rate, and decrease O_2^- level which damages the cells at excessive concentration (Yasui and Bava, 2006). This reaction is accompanied by alternate oxidation-reduction of metal ions present in the active site of SODs (McCord *et al.*, 1969; Tainer *et al.*, 1983).

O_2^- is continuously formed in aerobic organisms because of several biological processes which include aerobic respiration, oxidative phosphorylation, photosynthesis, substrate oxidation and during immune response of stimulated macrophages and neutrophils. Neutrophils and macrophages are the common phagocytes that use O_2^- against the attack of various pathogens (Fridovich, 1986; Johnston *et al.*, 1978). O_2^- and other ROS exist in biological systems at low but measurable concentrations (Sies, 1993). Their concentrations depend on the balance between their rates of production and their rates of clearance by various antioxidant systems. ROS plays a dual role as both beneficial and deleterious species, as they can be either beneficial or harmful to living systems (Valko *et al.*, 2006). Physiological concentration of ROS including O_2^- is sparingly advantageous for all organisms (Das, 1993). Various biochemical processes such as intracellular messaging during the cell differentiation and cell progression or in the arrest of growth and apoptosis (Ghosh and Myers, 1998) and defence by polymorphonuclear leukocytes against pathogens (Cleveland, *et al.*, 2000; Das, 1993), induction of mitogenic response are dependent on the low/moderate concentration of ROS. Unbalanced and elevated concentrations of ROS result in oxygen toxicity and are responsible for oxidative stress. Continuous oxidative stress may have significant adverse effects on the cell structure and functions, and may also bring about somatic mutations and neoplastic transformation in the cell (Fang *et al.*, 2009; Khandrika *et al.*, 2009).

Antioxidants play a key role in controlling cellular ROS levels and work not to remove oxidants entirely, but instead to keep them in a checked and balanced level

(Rhee, 2006). Antioxidants continuously scavenge ROS and include enzymatic as well as nonenzymatic molecules. SODs are very important antioxidant defence against oxidative stress in the body (Landis and Tower, 2005). Disruption of their functions are associated with several human diseases such as arteriosclerosis (Kakko *et al.*, 2003), diabetes mellitus (Haskins *et al.*, 2004; Ookawara *et al.*, 1992), Down syndrome (Engidawork and Lubec, 2001), etc. The high concentration of SOD is required to effectively compete for O_2^- in the presence of nitric oxide which is also present as an important biological molecule in the cell and reacts with O_2^- rapidly (Palmer *et al.*, 1987; Kissner *et al.*, 1998).

1.1.1. Types of SODs

Based on the metal cofactors present in the active sites, SODs can be classified into four distinct groups: Copper-Zinc SOD (Cu,Zn-SOD), Iron SOD (Fe-SOD), Manganese SOD (Mn-SOD), and Nickel SOD (Ni-SOD) (Miller *et al.*, 2001; Youn *et al.*, 1996). The different forms of SODs are unequally distributed throughout all biological kingdoms and are located in different subcellular compartments. The different forms of SODs are unequally distributed throughout all biological kingdoms and are located in different subcellular compartments. The Cu,Zn-SOD is found mainly in eukaryotes, chloroplast and bacteria (Sturtz *et al.*, 2001). Inside the eukaryotic cell, Cu,Zn-SOD occurs in the cytoplasm and outer mitochondrial space (Sturtz *et al.*, 2001). In addition, an extracellular Cu,Zn-SOD has been identified by some researchers in extracellular fluids including plasma, lymph and synovial fluid (Marklund *et al.*, 1982; Faraci & Didion, 2004). Mn-SOD is also found in all cells exposed to oxygen, from bacterial cells to humans. In bacteria, it is found in the cytosol, while in eukaryotes, it is found to be located in mitochondrial matrix (Abreu and Cabelli, 2010). In the aerobic halophyles, SODs are Mn-SODs (Cannio *et al.*, 2000), which have greater resemblance to the mitochondrial Mn-SODs, as compared to the bacterial Mn-SODs (Fink and Scandalios, 2002). Fe-SOD is found in both oxygenic as well as anoxygenic photosynthetic bacteria (Paumann *et al.*, 2002; Li *et al.*, 2002) and also in some green plants (Van Camp *et al.*, 1990; Miller, 2012). Ni-containing SOD has been isolated from a number of *Streptomyces* bacteria including *Streptomyces griseus* and *S. coelicolor* (Youn *et al.*, 1996; Kim *et al.*, 1996).

1.1.2. Cu,Zn-SOD

1.1.2.1. Occurrence

Gram-negative bacteria contain Cu,Zn-SOD in their periplasmic space (Gort *et al.*, 1999). This enzyme is not found in protists (Miller, 2012). Higher organisms such as plants, animals and fungi all possess Cu,Zn-SOD (Miller, 2012). In the yeast cell, Cu,Zn-SOD occurs in both cytoplasm and mitochondrial intermembrane space (Sturtz *et al.*, 2001). The Plants have been reported to possess multiple forms of Cu,Zn-SOD located in the cytosol and chloroplast (Miller, 2001; Van Camp *et al.*, 1990; Miller, 2012) and also in the peroxisomes (Bueno *et al.*, 1995), which are encoded by more than one gene. Animals have two different forms of Cu,Zn-SOD: dimeric cytoplasmic/intracelluar (McCord and Fridovich,1969) and tetrameric extracellular Cu,Zn-SOD (Antonyuk *et al.*, 2009). The intracellular Cu,Zn-SOD has also been reported in the nuclei, lysosomes and peroxisomes (Chang *et al.*, 1988). The extracellular Cu,Zn-SOD is distinct from the cytoplasmic one in terms of molecular mass well as the amino acid composition.

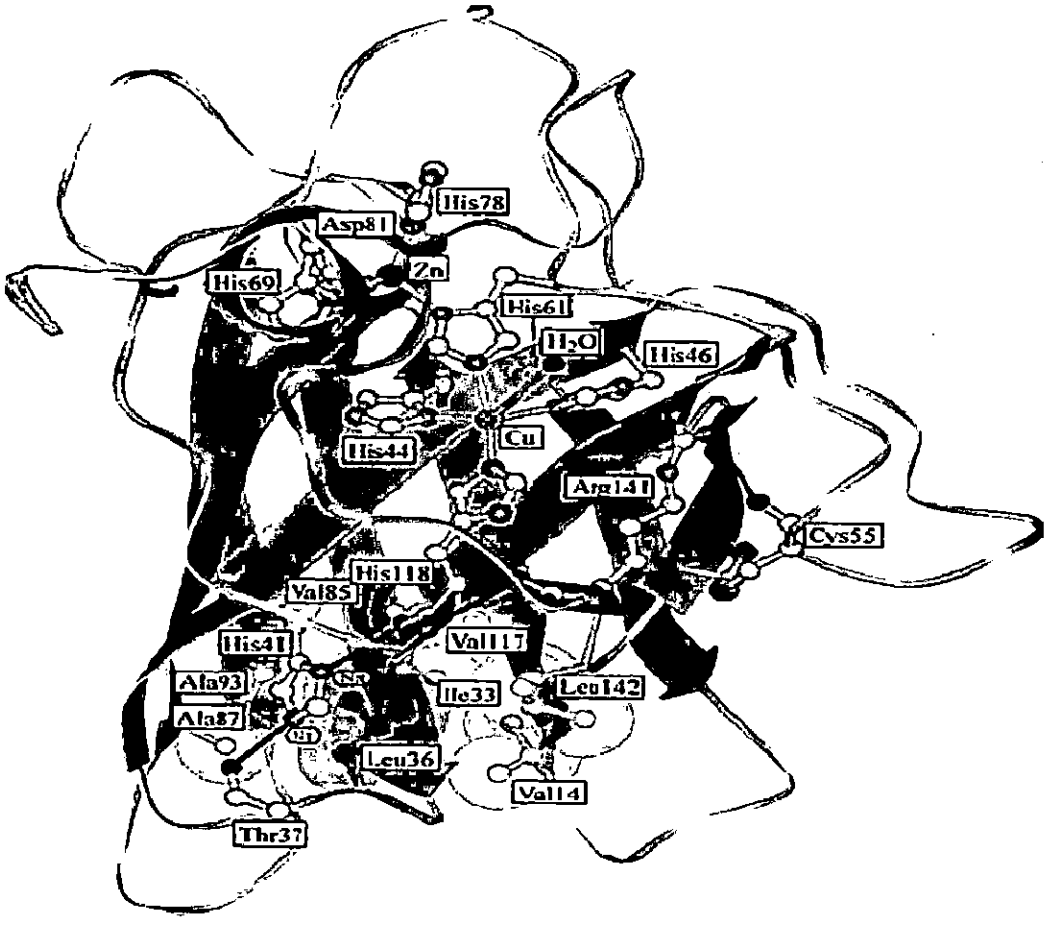
1.1.2.2. Bovine Cu,Zn-SODs

In 1982, the first complete three dimensional structure of Cu,Zn-SOD (Fig. 1) from the bovine erythrocytes was determined (Tainer *et al.*, 1982). Later in 1992, the structure of human Cu,Zn-SOD was solved (Parge *et al.*, 1992). It revealed that the human Cu,Zn-SOD shares high degree of sequence homology (83%), three dimensional structure, domain organization including amino acid sequence, protein fold and the catalytic properties with that of bovine erythrocyte enzyme (Bordo *et al.*, 1994; Didonato *et al.*, 2003). The structure of the eukaryotic enzyme is found to be highly conserved (Perry *et al.*, 2010).

Bovine Cu,Zn-SOD is composed of two identical subunits and has a molecular mass of 32.0 kDa (Hough and Husnain, 1999). Both subunits are related to each other by an approximate 2-fold symmetrical axis along the dimeric interface (Hough and Husnain, 1999). Extensive hydrophobic interactions and some hydrophilic contacts stabilize the dimer (Rae *et al.* 2001). Each subunit consists of a "greek key" eight stranded antiparallel β-barrel (Richardson, 1977) with three major external loops (Toyama *et al.*, 2002) (Fig. 2). The barrel contains 50% residues of the backbone (total 75 residues).

Fig. 1. Three dimensional structure of Cu,Zn-SOD. Cu,Zn-SOD is a dimer of two identical subunits each containing 150 amino acids. The enzyme active site contains a pair of Cu and Zn ions.

Fig. 2. Subunit structure of bovine Cu,Zn-SOD. The atomic coordinates were taken from those of subunit B in the X-ray crystal structure of Cu,Zn-SOD (Protein Data Bank Entry 2SOD). The side chains are shown for the residues in the active site, on the rim of the active site pocket, and at an opening of β-barrel near His 41. For the hydrophobic residues losing off the β-barrel opening, transparent balls with vander walls radii are superimposed on the ball and stick model. The central hydrophobic plug Leu 38 is coloured yellow. Blue thin sticks represent hydrogen bonds, and brown plates represent amide planes (Toyama *et al.*, 2004).


His78
Asp81
Zn
His69
His61
H_2O
His46
His44
Cu
Arg141
Cys55
His118
Val85
His41
Val117
Ala93
Ile33
Leu142
Ala87
Leu36
Val14
Thr37

The core of the barrel is tightly packed by hydrophobic residues (Richardson *et al.*, 1975; Perry *et al.*, 2010). The main chains inside the barrel, are separated by 12-13 Å in the shorter direction and 16-17 Å in longer direction. Another characteristic feature of the β-barrel is the presence of two halves. The first half, which is present towards the outside of the β-barrel, is very regular and less twisted. The second half is less regular and more twisted and is almost entirely internal to the subunit (Richardson *et al.*, 1975). A section of this barrel and two of the loops, electrostatic loop and zinc binding site form the walls of the active site (Li *et al.*, 2010). Electrostatic loop contains several charged residues which provide an electrostatic guidance for enzyme substrate interaction (Polticelli *et al.*, 1998; Ciriolo *et al.*, 2001; Getzoff *et al.*, 1992). The third loop provides a connection across the β-sheet. The active site is found to be located in a shallow pit, some 10 Å beneath the protein surface (Hough and Husnain, 1999). Each subunit contains one Cu and one Zn ion (Hough and Husnain, 1999). For the activity of the enzyme, active-site Cu ion is needed (DiDonato *et al.*, 2003; Gotto *et al.*, 2000), while both Cu and Zn ions contribute to the stability of the enzyme (DiDonato *et al.*, 2003; Forman & Fridovich, 1973; Abernethy *et al.*, 1974). In each subunit, Cu and Zn sites are separated by about 6 Å, forming a dimetallic catalytic centre. The Cu ion has three coordinate positions in reduced form in both aqueous and crystalline slurry and it adopts a trigonal planar geometry and is ligated by three histidine residues (His 44, His 46 and His 118) (Blackburn *et al.*, 1984). Cu ion is further found to be bridged with Zn ion by an anionic imidazole ring of another histidine residue (His 61) in its oxidized state. The Zn ion is ligated to one aspartic acid (Asp 81) and three histidines (His 61, His 69, His 78) and has a distorted tetrahedral geometry (Richardson *et al.*, 1975). There are eight histidine residues per subunit (Lippard *et al.*, 1977). NMR structural analysis clearly indicates that Cu ion is exposed to the solvent and Zn ion is buried inside (Gaber *et al.*, 1972; Cannio *et al.*, 2000). The bovine Cu,Zn-SOD has a disulfide bond (Tainer *et al.*, 1982; Getzoff *et al.*,1992) between the highly conserved pair of cysteines, namely Cys 55 and Cys 144 (Abernethy *et al.*, 1974). The binding of the active site metal ions and conserved disulfide bond in each subunit is considered to provide stability to the enzyme (Perry *et al.*, 2010).

The generally accepted catalytic mechanism for dismutation of O_2^- by SOD involves two steps: cyclic Cu reduction and reoxidation (Hart *et al.*, 1999). Two successive molecules of O_2^- are needed for this alternate reduction and oxidation of Cu ion.

$$O_2^- + Cu(II),Zn\text{-}SOD \longrightarrow O_2 + Cu(I),Zn\text{-}SOD \quad (1)$$

$$O_2^- + Cu(I),Zn\text{-}SOD + 2H^+ \longrightarrow H_2O_2 + Cu(II),Zn\text{-}SOD \quad (2)$$

The first step (equation 1) involves the reduction of Cu^{2+} and concominant Cu - N^{ε} - His 61 bond protonation. The protonation results in the loss of Cu - His 61- Zn bridge on the Cu site. However, a set of bovine Cu,Zn-SOD structures have been reported with an intact form of Cu-His61-Zn bridge (Banci *et al.*, 1994; Rypniewski *et al.*, 1995). Nevertheless, most of the data from solution studies strongly suggest the breakage of the imidazolate bridge. Reduction of Cu^{2+} takes place by inner sphere mechanism, and Cu^{2+} accepts electron directly from O_2^-. A trigonal planar coordination geometry is adapted by the resulting Cu^+ form (Bailey *et al.*, 1980; Murphy *et al.*, 1997; Hough and Hasnain, 2003). The product oxygen molecule easily diffuses out. The second step (equation 2) is suggested to proceed via outer sphere electron transfer from reduced Cu^+ to O_2^-. The electron transfer is followed by reoxidation of Cu^+, reformation of imidazolate bridge and release of H_2O_2 through proton donation from protonated His61. In both steps, O_2^- molecule is guided to the active site channel by a conserved set of charged amino acid residues (Getzoff *et al.*, 1989; Getzoff *et al.*, 1992). The nucleus of this mechanism is the formation of a stable complex between Cu and O_2^- molecule. Cu,Zn-SOD shows high catalytic efficacy. Pulse radiolysis studies have revealed that the catalysis is diffusion controlled and pH independent (pH 5-9.5) & $K_{cat} = K_1 = K_2 \approx 2\times10^9\ M^{-1}\ s^{-1}$ (Klug-Roth *et al.*, 1973; Argese *et al.*, 1987).

Cu, Zn-SOD is highly thermal stable enzyme and its dismutation ability starts to decrease above 80°C with a melting temperature (T_m) of 90°C (Roe *et al.*, 1988). The enzyme is quite resistant to denaturation by chemical treatment including 4% SDS and 10 M urea (Culotta *et al.*, 2006). It is also resistant to physical treatments such as heating, freezing and freeze–thaw cycles, and also cleavage by proteinase K (Bafana *et al.*, 2001). The enzyme is typically inhibited by azide, cyanide, diethyldithiocarbamate and H_2O_2, and is only gradually denatured in the presence of 6

M guanidine hydrochloride plus EDTA (Forman and Fridovich, 1973; Malinowski and Fridovich, 1979). The eight stranded antiparallel β-barrel, hydrophobic interactions associated with dimerization, co-ordinate/covalent bonds and an intrasubunit disulfide bond are considered to contribute to the remarkable stability of the enzyme (Hough and Hasnain, 2003; Perry *et al.*, 2010). The hydrophobic β-barrel core and main-chain β-sheet hydrogen bonds contribute both to the structural integrity and folding of the enzyme (Perry *et al.*, 2010).

In order to become enzymatically active, Cu,Zn-SOD undergoes several post translational modifications including the acquisition of Cu and Zn ions, formation of the disulfide bond and dimerization (Culotta *et al.*, 2006). The exact mechanism by which Cu,Zn-SOD acquires Zn^{2+} is not fully understood, there are some reports on the use of an accessory protein known as copper chaperone by the enzyme for facilitating the Cu insertion (O'Halloran and Culotta, 2000; Eisses *et al.*, 2000). Most of the cells employ the copper chaperone for SOD (CCS) to deliver and incorporate the Cu ion into Cu,Zn-SOD. CCS is a soluble Cu carrier protein which docks with and transfers the metal ion to the disulfide-reduced apo Cu,Zn-SOD (Culotta *et al.*, 1997).

The key characteristics of SOD include its exceptional stability, specificity of fold and assembly which are due to conserved features within the "Greek-key" β-barrel structure and dimer association as well as "cork" residues which stabilizes the ends of the β-barrel (DiDonato *et al.*, 2003; Getzoff *et al.*, 1989).

1.1.3. Physiological significance of SOD

SODs have great significance in the body as they catalyze the dismutation of O_2^- into H_2O_2 and oxygen molecule. The production of O_2^- occurs mostly within the mitochondria of the cell (Cadenas and Sies, 1998). The unchecked O_2^- may result in the formation of a cascade of deleterious ROS including H_2O_2, hypochlorite (OCl^-), peroxynitrite (ONO_2^-) and hydroxyl radical (HO^-) (Bryan *et al.*, 2012). While low levels of ROS have beneficial effects, the high levels may initiate many lethal reactions including inactivation of enzymes, damaging/alteration effects in DNA, damage of proteins, lipids etc. DNA mutations may lead to genomic instability and tumorigenesis (Wiseman and Halliwell, 1996). There are various human disorders clinically associated with oxidative stress (Rahman *et al.*, 2012). Therefore, SOD plays a critical role in protecting the organism against oxidative stress.

1.1.4. Therapeutic effects of SOD

Several studies have been performed that reveal the therapeutic potential and physiological importance of SOD (Noor *et al.*, 2002). The enzyme can serve as an anti-inflammatory agent and can also prevent precancerous cell changes (Yasui and Baba, 2006). Natural SOD levels in the body drop as the body ages (Inal *et al.*, 2001) and hence as one ages, one becomes more prone to oxidative stress related diseases. SOD is used in cosmetics and personal care products as an anti-aging ingredient and antioxidant because of its ability to reduce free radical damage to the skin, therefore preventing wrinkles, fine lines and age spots, and it also helps with wound healing, softens scar tissue, protects against UV rays, and reduces other signs of aging (Luisa Corvo *et al.*, 2002). It has been reported that SOD has an important link in several human health problems including RBC related disorders, cystic fibrosis, postcholecystomy pain syndrome, malignant breast disease, steroid sensitive nephrotic syndrome, amyotrophic lateral sclerosis, etc., neuronal apoptosis, AIDS and cancer (Noor *et al.*, 2002; Riley, 1999; Troy and Shelanski, 1994; Greenlund *et al.*, 1995; Riley, 1999; Bravard *et al.*, 1992; Church *et al.*, 1993; St. Clair *et al.*, 1994; Riley, 1999). Furthermore, a strong association between the activity of SOD and Alzheimer' disease has been suggested by some researchers (Noor *et al.*, 2002). It has also been reported that treatment with SOD helps recovery from mustard gas burns (Eldad *et al.*, 1998). In many animal models having myocardial ischemia-reperfusion injury, inflammation and cerebral ischemia reperfusion injury etc., SOD enzymes are found to be very effective (Salvemini and Riley, 2000). SOD mimics offer a potential for treating such diseases produced under conditions of oxidative stress (Riley, 1999). Several attempts have been made to use SOD as a therapeutic agent against the ROS mediated diseases.

Many SOD mimetics have been synthesized that can be used as pharmaceutical agents in a large number of diseases in which native SOD is ineffective (Salvemini *et al.*, 2002). Because of the instability, high immunogenicity and lesser circulation *in vivo* half-life of SOD, their clinical applications as therapeutic agent are very limited. To improve this pharmacological and biological problems, a wide variety of Cu,Zn-SOD conjugates have been developed with longer circulation half lifes, high stability and lesser immunogenicity (Ogino *et al.*, 1988; Kakimoto *et al.*, 1993). These SOD conjugates have exhibited marked effects *in vivo*.

1.1.4.1. SOD and cancer

SOD, being a key cellular antioxidant, is highly responsible for the elimination of $O_2^{\cdot-}$. The inactivation and/or deficiency of SOD results in the irreversible cellular injury, which is mediated by accumulation of $O_2^{\cdot-}$. Many studies have revealed the critical role of oxidative stress in carcinogenesis (Wiseman and Halliwel, 1996; Monya *et al.*, 2001). Indeed, there are several clear evidences indicating that ROS works as an endogenous class of carcinogens by inducing mutations in the cells (Guyton and Kensler, 1993; Feig *et al.*, 1994; Gerutti, 1994). The diminished activity of Cu,Zn-SOD and Mn-SOD has been observed in cancer cells (Bafana *et al.*, 2011; Oberley, 2004; St. Clair, 2014). Normalization of SOD level contributes to part of the cancer cell phenotype reversion (Bafana *et al.*, 2011). It has been suggested that SOD may regulate cancer progression and hence, can be used as a novel target for cancer treatment (Papa *et al.*, 2014a; Papa *et al.*, 2014b; Tsang *et al.*, 2014; Glasauer *et al.*, 2014). Furthermore, It has been shown that Cu,Zn-SOD can be used as a novel therapeutic target for the treatment of multiple myeloma (Salem *et al.*, 2015). On the contrary, the invasive and migratory activity of pancreatic cancer is encouraged by SOD via activation of the H_2O_2/ERK/NF-κB axis (Li *et al.*, 2015).

1.1.4.2. SOD and inflammatory diseases

Neutrophils play a central and essential role in the pathogenesis of inflammation. Activated neutrophils adhere to vascular endothelium and trans-migrate to the extravascular space, release ROS, protease enzymes, and large amounts of chemokines (Yasui and Baba, 2006). ROS and proteases damage normal tissue and extracellular matrix proteins. $O_2^{\cdot-}$ serve to activate endothelial cells and enhance neutrophil infiltration (Salvemini *et al.*, 1999; Masini *et al.*, 2002). Studies performed in transgenic mice overexpressing extracellular SOD (Ghio *et al.*, 2002) and SOD mimetic (Salvemini *et al.*, 1999) have shown that inhibition of $O_2^{\cdot-}$ can prevent the infiltration of neutrophils at the site of damage. Neutrophil apoptosis may also be important step in the resolution of inflammation. In individuals with down syndrome, neutrophil apoptosis increases and Cu,Zn-SOD is overexpressed (Yasui *et al.*, 1999). Exogenous H_2O_2 together with SOD, increase the number of apoptotic neutrophils (Yasui *et al.*, 2005). SOD may serve as an inhibitory agent of neutrophil mediated inflammation and may stand for a novel therapeutic approach for the ROS dependent

tissue damage induced by neutrophils via several mechanisms (Yasui and Baba, 2006). Preclinical studies with bovine Cu,Zn-SOD showed encouraging results for its use as a human therapeutic agent in acute and chronic inflammatory conditions, including dermatosis due to burn and wound injury (Flohe, 1988; Niwa, 1989). Extracellular SOD, Mn-SOD and Cu,Zn-SOD have been described as potential inhibitor of inflammation by various reporters (Bowler *et al.*, 2004; Joseph *et al.*, 2008).

1.1.4.3. SOD and cystic fibrosis

Cystic fibrosis (CF) is characterized by the chronic inflammation, and the recruitment of activated neutrophils (De Rose, 2002). In the plasma of patients with CF, SOD activity was significantly lower as compared with the healthy individuals (Madarsi *et al.*, 2000). Also, in mononuclear, polymorphonuclear and red cells of CF patients, reduced Cu,Zn-SOD activity was observed (Percival *et al.*, 1995). It has been found that the antifibrotic action of Cu,Zn-SOD is mediated by TGF-β1 repression followed by phenotypic reversion of myofibroblasts (Vozenin-Brotons *et al.*, 2001). Radiation-induced fibrosis of breast was significantly reduced by Cu,Zn-SOD (Campana *et al.*, 2004). These findings indicate new therapeutic possibilities targeting antioxidant pathways including SOD, so that oxidative stress and apoptosis can be reduced in CF cells, and proinflammatory response can be limited.

1.1.4.4. SOD and ischemia

ROS including O_2^- and its reaction product, peroxynitrite have a significant role in endothelial and tissue injury associated with ischemia and reperfusion. Over expression of Cu,Zn-SOD reduces ischemic damage resulting from ischemia/reperfusion (Yang *et al.*, 1994). Mn-SOD targeted deletion deteriorates the outcome from both temporary and permanent middle cerebral artery occlusion (Kim *et al.*, 2002; Murakami *et al.*, 1998). The removal of O_2^- and peroxynitrite by SOD mimetic helps in the prevention of cellular energetic failure and tissue damage related with ischemia and perfusion and has a beneficial effect in this situation (Salvemini and Cuzzocreas, 2002).

1.1.4.5. SOD and aging

SOD is considered to be an antiaging enzyme. The free radical theory of aging was proposed by Derham Harman (Harman, 1956). It postulated that oxygen free radicals generated in metabolic pathways, causes age related deterioration through oxidative damage to biomolecules with mitochondria being the main target of attack. Accumulation of oxidative damage is considered to be one of the key mechanisms of aging (Harman 1956; Hekimi and Guarente, 2003; Longo and Finch, 2003).

Drosophila flies having 75% reduction in SOD activity, showed accelerated loss of olfactory behaviour upon ageing (Paul *et al.*, 2007). The loss of SOD2 activity in *Drosophila* is highly related to the life span and age-related decline of olfactory system function. Studies on a series of *Drosophila* SOD2 mutants reveal the essential role of this gene in protecting flies from mitochondrial oxidative damage, neurodegeneration, age-related defects in behaviour and early-onset mortality (Paul *et al.*, 2007).

1.1.4.6. SOD and rheumatoid arthritis

Rheumatoid arthritis is a systemic disease and is characterized by a chronic inflammation reaction in the synovium of joints leading to degeneration of cartilage and erosion of juxta-articular bone. ROS and other free radicals play an important role in the inflammation process and also damage cartilage and the extracellular matrix (Mahajan and Tandon, 2004; Hitchon and El-Gabalaway, 2004). Increased oxidative stress or deficient antioxidant status are critical in the pathogenesis of rheumatoid arthritis (Mahajan and Tandon, 2004; Hitchon and El-Gabalaway, 2004). Some antioxidants including SOD and vitamin E have an anti-inflammatory role in experimentally induced arthritis (Mahajan and Tandon, 2004). It was found that SOD activity is low in patients suffering from rheumatoid arthritis and the administration of SOD through liposomes had a positive effect in the treatment of experimental arthrits (Uger *et al.*, 2004; Karatas *et al.*, 2003).

1.1.4.7. SOD and neurodegenerative diseases

Oxidative stress has been shown to be involved in the pathophysiology of several neurodgenarative diseases. The affected regions of patients having Alzheimer disease have reduced activity of antioxidant enzymes such as SOD, catalase,

glutathione peroxidase and glutathione peroxidase (Zemlon *et al.*, 1989; Papolla *et al.*, 1992). Familial amyotrophic sclerosis (FALS), is a fatal neurodegenerative disease that leads to the selective loss of motor neurons. Several mutations in Cu,Zn-SOD gene are found to be associated with FALS (Cleveland and Rothstein, 2001). In addition, Cu,Zn-SOD is one of the prime victim of oxidative damage to the brain in Alzheimer disease and Parkinson disease (Choi *et al.*, 2005).

1.1.4.8. SOD and diabetes

Increased oxidative stress plays a major role in the aetiology of diabetes and its complications (Baynes, 1991; Baynes and Thorpe, 1999; Ceriello, 2000). In diabetes, persistent hyperglycemia stimulates the production of ROS from various sources (Yan, 2014). As a result, diabetes usually leads to increased formation of ROS and weakened antioxidant defences (McLellan *et al.*, 1994; Saxena *et al.*, 1993). The enhanced production and/or decreased removal of ROS by nonenzymatic and enzymatic antioxidant defences, increases the level of ROS in diabetic patients which damages many tissues, resulting in diabetic complications (Lipinski, 2001).

Glyoxidation hypothesis states that glyoxidation plays a leading role in damage of long lived proteins during diabetes and aging (Baynes and Thorpe, 1999). The α-ketoaldehyde formed from the auto-oxidation of glucose might significantly contribute to the commencement of nonenzymatic glycosylation of proteins (Wolff and Dean, 1987). Glucose auto-oxidation appears to be one of the main sources of free radicals during hyperglycemia (Ceriello, 2000). Enediol form of glucose is readily oxidized to enediol radical anion in a transition metal dependent reaction. Further, the enediol radical becomes converted into reactive ketoaldehydes and $O_2^{\cdot}$. SOD catalyses the conversion of $O_2^{\cdot}$ into H_2O_2. If H_2O_2 is not degraded by catalase and peroxidase, it can lead to formation of highly reactive $HO^{\cdot}$. Moreover, reducing sugars such as glucose can also be oxidized and can form low molecular mass dicarbonyl compounds. These resultant dicarbonyl compounds are highly reactive and may react directly with the side chains of amino acid residues including both arginine and lysine and lead to the formation of a wide range of protein bound advanced glycation endproducts (AGEs) and crosslinks (Thorpe and Baynes, 2003).

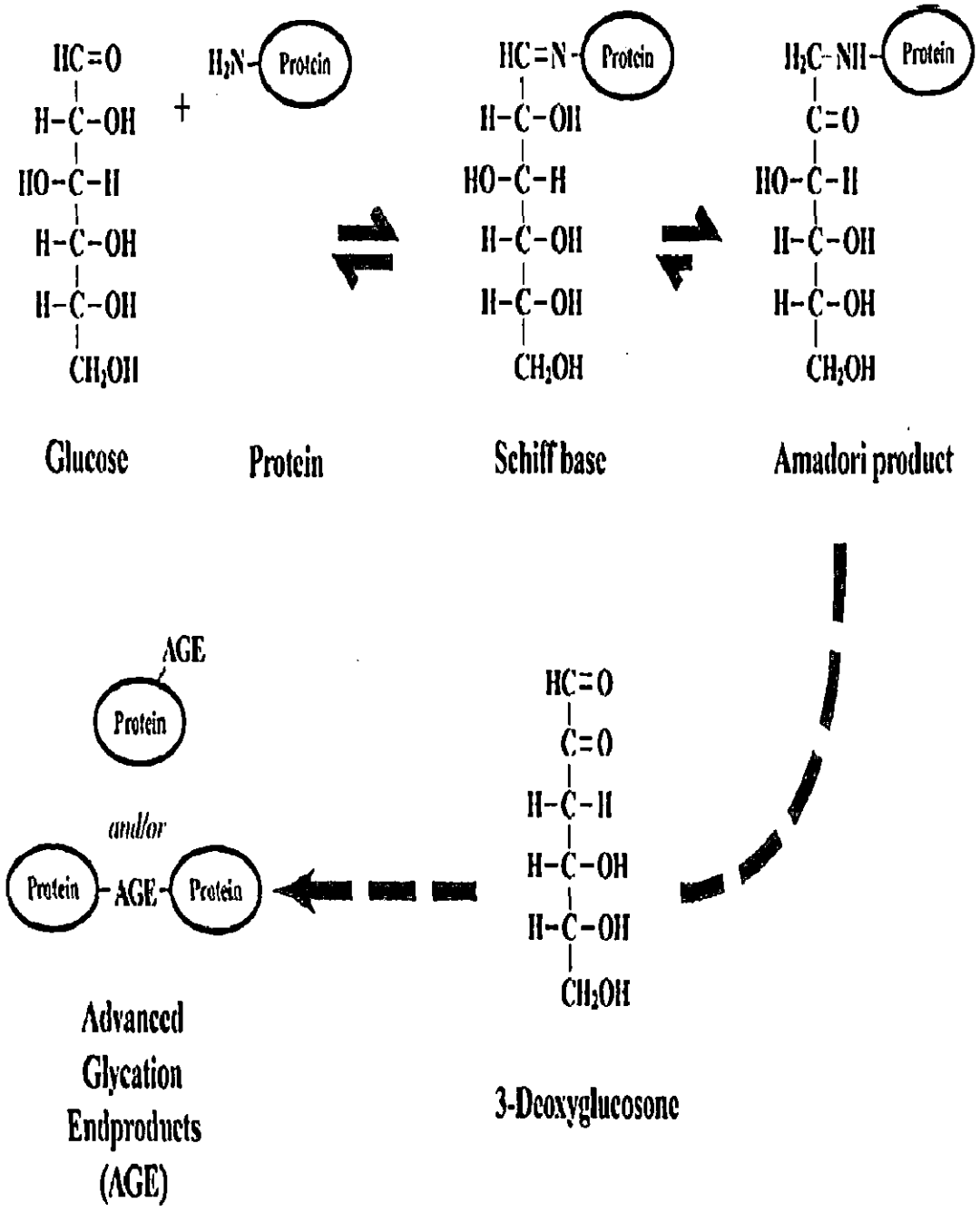
Under hyperglycemic conditions, endothelial cells produce elevated levels of $O_2^{\cdot-}$ (Graier *et al.*, 1999). Overproduction of $O_2^{\cdot-}$ can inhibit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is an important enzyme of the glycolytic pathway (Du *et al.*, 2003). This leads to the accumulation of glucose and other intermediate metabolites of this pathway and shifts to other alternative pathways of glucose metabolism along with increased production of AGEs. The enormous amount of $O_2^{\cdot-}$ causes the state of oxidative stress, which in turn causes the impaired antioxidant defense, increased glucose auto-oxidation and protein glycoxidation (Baynes, 1991). The oxidation reaction of both free and protein-bound sugars mediated by radicals, is termed as glycoxidation (Pennathur and Heinecke, 2004). Protein glycoxidation indicates the underlying-chemical modification and crosslinking of tissue protein that is induced by reducing sugars and glycoxidation products can be regarded as the biomarkers of carbohydrate dependent damage of proteins (Baynes, 1991).

Lipid peroxidation of low density lipoproteins is also found to be promoted by hyperglycemia (Jain, 1989). Glycation or non-enzymatic glycosylation of proteins is also a source of free radicals in diabetes, contributing to diabetic complications (McCance *et al.*, 1993). Further, increased oxidative stress is found to be associated with diabetic complications (Kangralkar *et al.*, 2010; Ceriello, 2000).

1.2. Glycation or non-enzymatic glycosylation of proteins

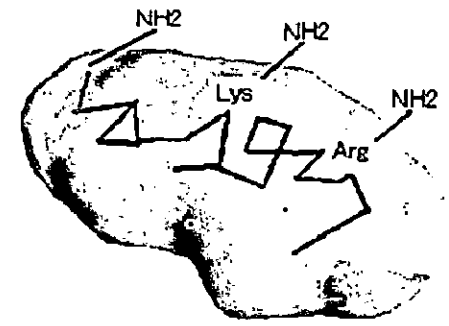
Glycation or Maillard reaction is a spontaneous, naturally occurring, non-enzymatic and complex network of reactions which is initiated by reaction of carbonyl group of a reducing sugar (e.g., glucose, galactose, fructose, mannose or ribose) with a free amino group, typically the ε-amino group of lysine residues and the α-amino group at the N-terminus of a protein to form an adduct commonly referred to as the Schiff base (Zhang *et al.*, 2009) (Fig. 3). The Schiff bases undergo Amadori rearrangement and through a series of further rearrangement, cyclizations etc. form a variety of diverse compounds collectively described as AGEs (Neglia *et al.*, 1983; Baynes *et al.*, 1989). AGE formation is accompanied by the formation by the formation, among others of a number of ROS, α-oxoaldehydes, that further react and damage the proteins and other important biological molecules. Glycation plays an important role in the development of physiological and pathophysiological processes such as aging, diabetes, atherosclerosis, neurodegenerative diseases and chronic renal failure (Brownlee, 1995).

Fig. 3. Glycation of proteins. The initial reaction between glucose and protein amino groups forms a reversible Schiff base that rearranges to a ketoamine or Amadori product. With time, these Amadori products form AGEs via dicarbonyl intermediates.


HC=O
H-C-OH
HO-C-H
H-C-OH
H-C-OH
CH2OH
H2N-Protein
HC=N-Protein
H2C-NH-Protein
C=O
Glucose
Protein
Schiff base
Amadori product
AGE
Protein
and/or
Protein-AGE-Protein
3-Deoxyglucosone
Advanced Glycation Endproducts (AGE)

The Maillard reaction was first observed by Louis-Camille Maillard in 1912 (Maillard, 1912). Upto 1980's, most of the work on glycation was done for the food science field and related to the flavour, colour and nutritional value of food. Nowadays, pathophysiological impacts of the Maillard reaction on the human health are more of concern in relation to the diabetic complications, and age related diseases such as atherosclerosis, Alzheimer's disease and inflammation (Basta *et al.*, 2002; Baynes and Thorpe., 1999; Munch *et al.*, 1998). In the physiological systems, cellular and extracellular proteins are spontaneously damaged by glycation (Thornalley, 1999; Thornalley *et al.*, 2003). Due to glycation, not only the structural properties such as charge, solvation and/or conformation of proteins, but also the biological properties are affected (Fig. 4). Therefore, glycation can also change the functional properties of proteins (Darewiczb and Dziuba, 2001; Khan *et al.*, 1999; Morgan *et al.*, 1999; Nakamura *et al.*, 1994). The resulting protein modification can possibly explain the reason for the tissue damage that occurs in persistent hyperglycemic conditions (Dyer, 1993; McCance *et al.*, 1993). Furthermore, glycation can induce structural changes in enzymes that include conformational alterations, thio-oxidation, aggregation, formation of disulphide and other cross links, and inactivation of enzymes (Harding, 1991). Previous studies have reported that glycation causes the alteration in the activity of some enzymes such as SOD (Arai *et al.*, 1987a; Arai *et al.*, 1987b), carbonic anhydrase (Kondo *et al.*, 1987), alcohol dehydrogenase (Shilton and Walton, 1991), aldehyde reductase (Takashaki *et al.*, 1995), etc. Although, a number of free amino groups are present on the surface of proteins, only a few are selected for glycation. Only those amino groups are selected for glycation that are either adjacent to an imidazole moiety or are a part of a lysine doublet. If an amino acid is very close (approximately 5Å) to an imidazole group, it will be highly susceptible to glycation (Bunn *et al.*, 1976). The degree of glycation of a protein depends on a number of independently acting factors such as pH, temperature, protein concentration, etc. (Brownlee *et al.*, 1984, Eble *et al.*, 1983). In addition, it is highly affected by the glucose concentration as well as the incubation period, which are both clinically more relevant factors (Brownlee *et al.*, 1984). The rate of glycation in vivo depends on the sugar concentration, reactivity of the free amino groups of the biomolecules and their half-life (Nawale et al., 2006).

Fig. 4. Effect of glycation on proteins and AGE formation. A nonenzymatic reaction between glucose or other reducing sugar and the N-terminal amino acid residues and/or ε-amino groups of proteins initially forms a Schiff base adduct. The Schiff base adduct then slowly undergoes Amadori rearrangement. Additional dehydration, condensation, fragmentation, rearrangement, and oxidation results in the formation of AGE (Kikuchi *et al.*, 2002).

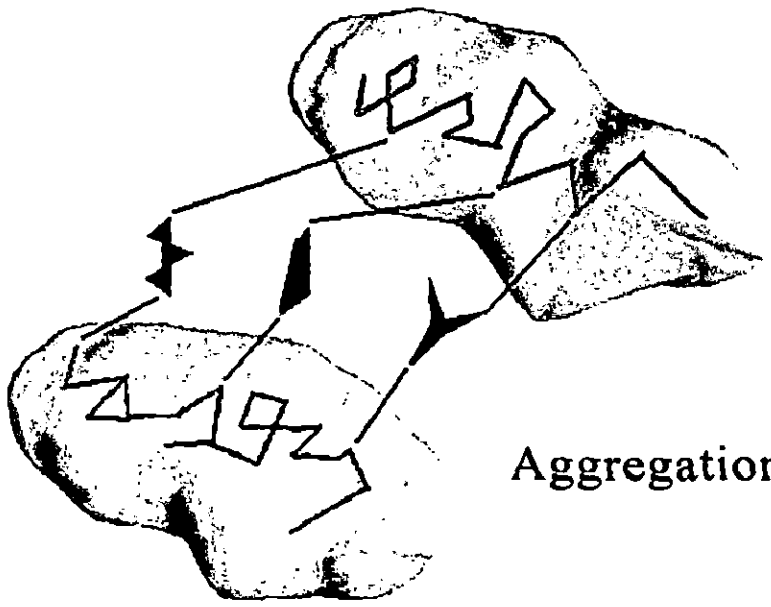


Glycation → ROS production

Loss of function

Accelerated degradation

Cross Linkage



Aggregation

AGEs

1.2.1. Chemistry of glycation

Glycation is a non-enzymatic covalent interaction between reducing sugars and either ε-amino group of lysine residues or α-amino group present at the N-terminal groups of a protein. It is an example of neucleophilic addition reaction (Dyer, 1993; Forbes *et al.*, 2003; McCance *et al.*, 1993; Price *et al.*, 2001; Ulrich and Cerami, 2001). It can be divided into three prime steps: early, intermediate and late. In the early stage, glucose or other reducing sugars react with the nucleophilic groups of proteins, lipids, and nucleic acids and form Schiff base as the intermediate products (Ahmed, 2005; Brownlee *et al.*, 1984). Formation of Schiff base is relatively very fast and highly reversible. Schiff bases are highly unstable and are reversible aldimine compounds (Brownlee *et al.*, 1984; Ulrich and Cerami, 2001). Depending on the surrounding glucose concentration, Schiff base rapidly reaches an equilibrium level *in vivo*. After several rearrangements, Schiff base leads to the formation of a stable yet reversible ketoamine or Amadori products. The rate of production of Amadori products is dependent on the concentration of glucose (Brownlee, 1995). They become accumulated on both short lived as well as long lived proteins (Thornalley, 1996). During the formation of AGEs, Amadori products are intermediate reaction products (Brownlee *et al.*, 1984; Baynes *et al.*, 1999; Ahmed, 2005; Ulrich and Cerami, 2001).

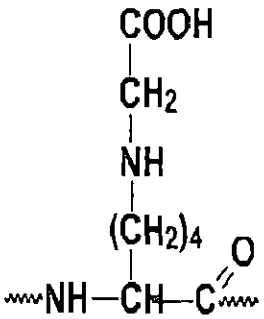
In the intermediate stage, the degradation of Amadori products leads to formation of a number of carbonyl compounds, such as glyoxal, methylglyoxal (MG) and deoxyglucosones that may work as propagators of the reaction (Thornalley *et al.*, 1999; Glomb and Monnier, 1995). Amadori products can undergo several transformations through numerous divergent pathways. These pathways can involve oxidation, fragmentation, enolization, dehydration, acid hydrolysis and free radical reactions, and result in the formation of a large variety of poorly characterized compounds (Friedman, 1996). In the late or final step, the propagators further react with free amino groups and form AGEs after undergoing various reactions including irreversible oxidation, dehydration and cyclization etc. AGEs are yellow-brown heterogeneous compounds with an ability to form covalent bonds with the amino groups of other proteins and thus cause protein cross-linking (Eble *et al.*, 1983). They can disturb several physiological functions because of their ability to form insoluble aggregates. AGEs are considered to be relatively indigestible. However, AGEs may

be taken up to lysosomes via some specific cell-surface binding sites, the receptors for AGE (RAGE), as well as macrophage scavenger receptor, where they are degraded out into small AGE peptides or fragments (Vlassara *et al.*, 1994). The resultant fragments can contain irregular components of parental AGE moieties (Vlassara *et al.*, 1994).

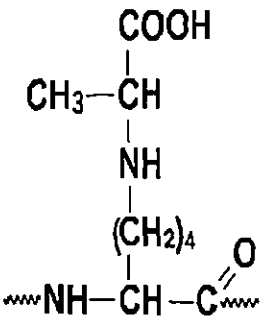
1.2.2. Pathogenicity of AGEs and glycation

In vivo AGEs accumulate in the plasma (Makita *et al.*, 1995), vascular tissues (Aronson, 2002), the kidney (Heidland *et al.*, 2001) and on arterial wall (Brownlee *et al.*, 1986). AGEs are supposed to be involved in the pathogenesis of age-related disorders that can affect connective tissue, lens, blood vessels, and nerves (Brownlee, 1995). Previously, it was thought that only long lived extracellular proteins accumulate AGEs, however it is now known that they can be formed on short-lived molecules and even intracellular growth factors (Giardino *et al.*, 1994). Extracellular proteins are more likely to be influenced by AGE modification because of slow turn-over rate (Goh and Cooper, 2008). Glycation leads to intra-molecular as well as inter-molecular crosslinking with collagen. Crosslinking brings about several structural alterations which result in the increased stiffness as well as resistance to proteolytic digestion (Goh and Cooper, 2008). These processes are increased in the persons suffering from diabetes. AGEs continuously accumulate over the lifetime of the protein and do not return back to their normal when hyperglycemia is corrected. It is suggested that even a moderate elevation in blood glucose level can result in a substantial increase in AGE accumulation (Ahmed, 2005). The major AGEs (Fig. 5) *in vivo* appear to be produced from highly reactive intermediate carbonyl groups, known as α-dicarbonyls or oxoaldehydes, including 3-deoxyglucosone, glyoxal, and MG (Brownlee, 2001; Thornalley, 1996). The rate of intracellular AGE formation is faster as compared to that in the extracellular compartment (Monnier, 1989) because the rate of glycosylation product formation is found to be slowest for glucose as compared to all naturally existing sugars (Brownlee, 1995). Glycation *in vivo* along with AGEs accumulation has been found to be implicated in the pathogenesis of several chronic diseases including typical diabetic complications, atherosclerosis, Alzheimer's disease, rheumatoid arthritis and chronic heart failure (Smit *et al.*, 2008; Meerwaldt *et al.*, 2008; Smit and Lutgers, 2004; Vlassara and Palace, 2002).

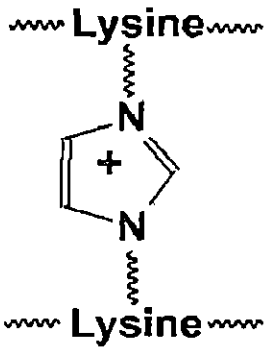
Fig. 5. Chemical structures of various major protein AGEs detected in tissue proteins. (Thorpe and Brown, 2003).



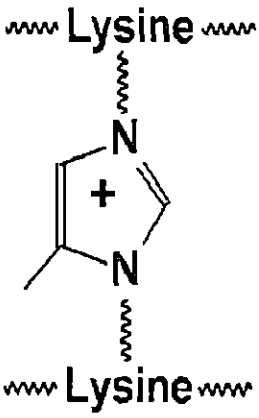
CML



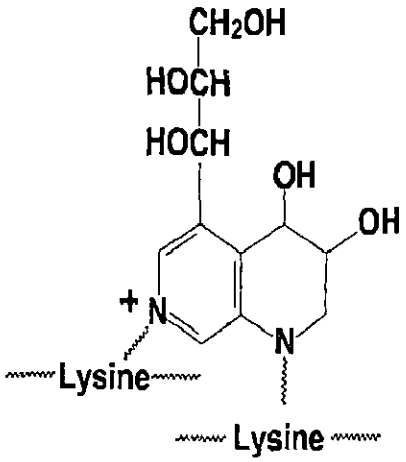
CEL



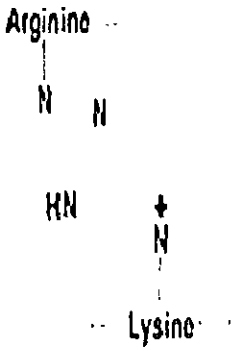
GOLD



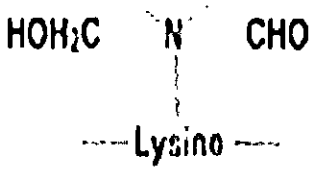
GOLD



Crosslines



Pentosidine



Pyrraline

Increased glycation and build-up of tissue AGEs can alter enzymatic activity, decrease ligand binding, modify protein half-life and alter immunogenicity, so they have been considered to be a major factor contributing to diabetic complications (Vlassara and Palace, 2002). Furthermore, increased free radical production during protein glycation and AGE formation is also a major cause of biomolecular damage in diabetic conditions (Ahmed, 2005; Wautier and Guillausseau, 2001). Atherogenesis in diabetic patients is caused by AGE-immune complexes formed from autoantibodies and their corresponding serum AGEs (Turk *et al.*, 2001). Several AGEs are more chemically reactive and are termed as glycotoxins (Vlassara *et al.*, 1994). During diabetes and ageing, one of very common symptoms is the rigidity as well as thickening of capillary walls, which is caused by cross-linking of collagen proteins by AGEs (Nawale *et al.*, 2006).

Free radicals formed during glycation, can lead to protein fragmentation and oxidation of nucleic acids and lipids (Baynes, 1991). Moreover, glycation cytotoxicity can be a result of production of ROS, inhibition of specific functions of proteins, cross linking, aggregation, and precipitation of proteins. Reduced enzymatic activities of Cu,Zn-SOD and other antioxidant enzymes have been reported to occur in the kidney, heart tissue as well as the liver of diabetic patients, while the levels of ROS including O_2^- are increased (Asayama *et al.*, 1989; Giugliano,1995).

The pathogenic effects of AGEs are exerted either directly or by interaction with some specific receptors e.g. RAGE. RAGE is found on smooth muscle cells, macrophages, endothelial cells and astrocytes (Ahmed, 2005). RAGEs are very crucial as their interaction with AGEs induces the activation of secondary messenger pathways such as protein kinase C. The binding of ligand with RAGE triggers the activation of the NF-κB and other signalling pathways via the stimulation of extracellular signal-regulated kinase-1/2 (ERK), p38 MAPK (mitogen-activated-protein-kinase), the stress-activated protein kinase/c-jun Nterminal kinase (SAPK/JNK) kinases, rho-GTPases, phosphoinositide 3-kinases, JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway and Rac-Cdc42 (Bopp *et al.*, 2008). Several of them are the results as well as the cause of ROS (Thornalley, 1998; Hori *et al.*, 1995; Sorci *et al.*, 2004; Taguchi *et al.*, 2000; Huang *et al.*, 2001; Ishihara *et al.*, 2003). During inflammation and diabetes, RAGE is highly expressed. Some researchers have found that pharmacological blockade of RAGE

in db/db diabetic mice provides protection against glomerulosclerosis and early diabetic nephropathy (Wendt *et al.*, 2003). Deletion of the RAGE gene protects animals from the adverse effects of diabetes, while over expression of RAGE supports diabetic neuropathy (Toth *et al.*, 2008; Vincent *et al.*, 2007). Therefore, RAGE has a considerable role in diabetic nephropathy.

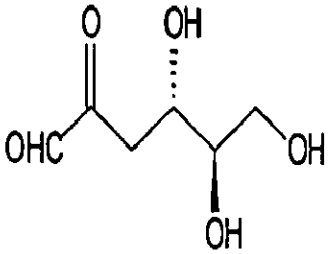
1.2.3. Dicarbonyls formed during glycation

Prolonged hyperglycemia exerts many detrimental effects and may be the key cause of most diabetic complications. Because of hyperglycemia, numerous dicarbonyl compounds become accumulated that lead to carbonyl stress. Dicarbonyls, in general, can be produced as glycolytic intermediates during glucose metabolism and as secondary intermediate products produced during Maillard reaction by degradation of glycated proteins as well as during the lipid peroxidation of polyunsaturated fats (Turk, 2010). Moreover, the sugar moiety, itself can lead to formation of several low molecular weight dicarbonyl compounds such as MG, glyoxal, glycoaldehyde, 3-deoxyglucosone (3-DG), etc (Fig. 6 and Fig. 7). The plasma from diabetic patients has high level of these dicarbonyls (Han *et al.*, 2007). The dicarbonyl compounds are even more reactive than their parent sugars and act as key components of carbonyl stress (Thorpe *et al.*, 2000). They are characterized by remarkable high chemical reactivity and are able to form AGE-structures even at very low concentration. They have been suggested to have a role in the carbonyl stress reactions leading to hasten vascular damage in diabetes (Thorpe *et al.*, 2000).

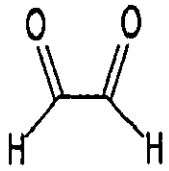
MG or pyruvaldehyde is a highly reactive electrophilic α-oxaldehyde or dicarbonyl compound (Thornalley, 1996; Kalapos, 1999) and is generated through both enzymatic as well as non-enzymatic pathways intracellularly. Glyoxalase checks its interactions with body protein and nucleic acids (Thornalley, 1999) and converts it into the inert product D-lactate (Thornalley, 1994a; Thornalley, 1994b). MG is produced either from the fragmentation of an ene-diol triose phosphate glycolytic intermediates [dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate] or by the spontaneous elimination of a phosphate group from glyceraldehyde-3-phosphate and DHAP (Thornalley, 1999). Rate of MG formation becomes high during diabetes and especially in the cells having high amount of accumulated glucose during hyperglycemia (Turk, 2010; Brownlee, 2001).

Fig. 6. Structures of some intermediate dicarbonyls formed during glycation.

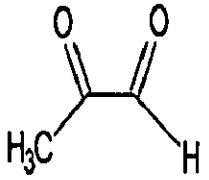
Fig. 7. Mechanistic interpretation of glyoxal, methylglyoxal and 3-deoxyglucosone formation in early glycation. RNH_2 represents lysyl side chain and N-terminal amino groups (Thornalley *et al.*, 1999).



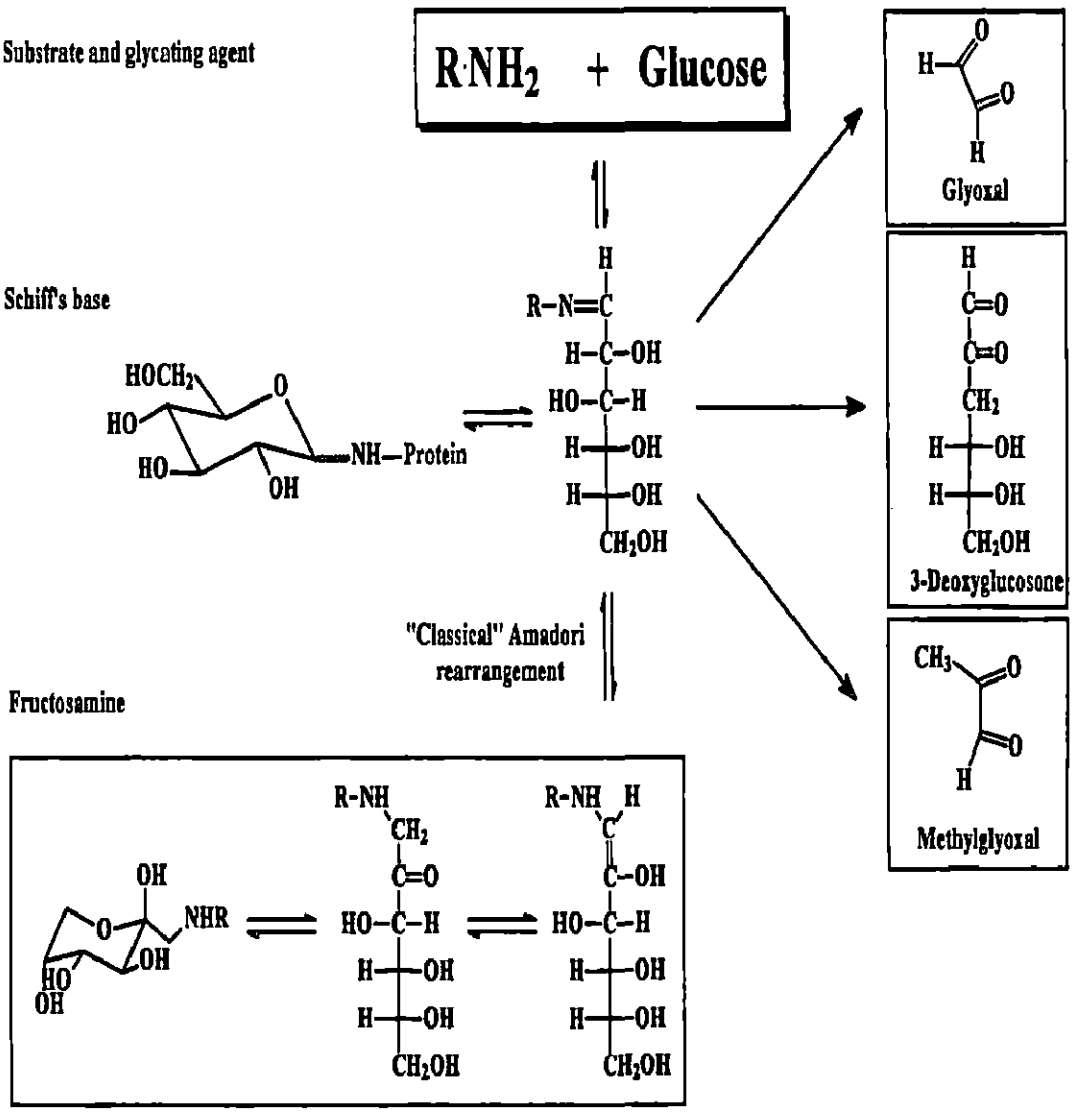
3-Deoxyglucosone (3-DG)



Glyoxal (GO)



Methylglyoxal (MGO)



MG synthase is believed to be involved in the conversion of DHAP into MG, especially when the supply of inorganic phosphate is inadequate (Hopper and Cooper, 1971). In addition, MG is also formed from ketone body metabolism, oxidation of acetoacetate, oxidation of acetoacetate in neutrophils catalyzed by myeloperoxidase and catabolism of threonine via aminoacetone in diabetic ketoacidosis (Thornalley, 1999; Lyle and Singh, 1992).

MG is a more potent glycating agent as compared to because the specific reactivity of MG is 20,000-fold higher than that of glucose (Rabbani and Thornalley, 2014). The concentration of MG in plasma is approximately 50,000-fold lower than that of glucose because glyoxalase enzyme is efficiently able to detoxify MG *in vivo* (Rabbani and Thornalley, 2014). Diabetes and other metabolic disorders can increase the rate of formation of MG (Nagaraj *et al.*, 1996). As a consequence, the concentrations of MG and MG-derived AGEs are found to be increased in tissues (Ahmed, 1997). There can also be another cause for arise in MG level such as decreased clearance of MG by the detoxification pathways. MG plays an important role in oxidative stress (Desai *et al.*, 2008). Several studies have indicated that MG increases the production of O_2^- (Chang *et al.*, 2005; Ho *et al.*, 2007), H_2O_2 and ONO_2^- (Chang *et al.*, 2005; Dhar *et al.*, 2008; Ward *et al.*, 2004), proinflammatory cytokines such as interleufkin 1 (IL-1) (Di Loreto *et al.*, 2004), IL-6 and IL-8 (Wang *et al.*, 2007; Di Loreto *et al.*, 2004).

Carboxyethyl lysine (CEL) and MG lysine dimer (MOLD) are generated when MG reacts with lysine residues of proteins (Bourajjaj *et al.*, 2003). However, being an arginine-directed glycating agent, MG mainly forms a hydroimidazolone adduct, MG-H1 (Rabbani and Thornalley, 2012). Therefore, proteins are more like to be functionally altered by MG glycation because their functional domains have higher number of arginine than lysine residues (Thornalley and Rabbani, 2011). Besides, MG reacts rapidly with functional groups of proteins such as guanidino and thiol groups, and leads to browning and crosslinking of proteins as well as formation of fluorescent products, and it ultimately can cause denaturation of proteins (McLaughlin *et al.*, 1980; Lo *et al.*, 1994). Both, cellular and extracellular proteins can be glycated by MG because a minor fraction of intracellularly formed MG leaks out and bring about the glycation of extracellular proteins (Karachalias *et al.*, 2010).3-DG, a small aliphatic aldehyde with a low molecular mass and it takes part

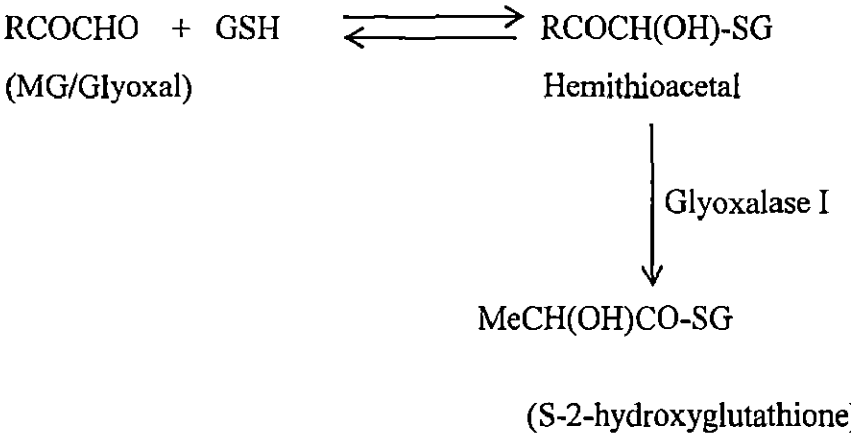
in the formation of adducts with proteins. The production of 3-DG in humans occurs either during the intermediate steps of Maillard reaction or from fructose-3-phosphate through the polyol pathway (Turk, 2010). Studies have shown the presence of 3-DG in the lens and heart of diabetic rats, and elevated level of 3-DG occurs in the erythrocytes of haemodialysis patients (Lal *et al.*, 1997; Tsukushi *et al.*, 1999). Several 3-DG related AGEs such as caboxymethyl lysine (CML), 3-DG-imidazole, pyrraline and a minor product "pentosidine" are produced only when 3-DG reacts with the amino groups of proteins (Jono *et al.*, 2004). 3-DG-imidazolone is a dominant product of 3-DG derivatived AGEs (Ahmed *et al.*, 2005; Jono *et al.*, 2004). Like MG, 3-DG has the ability to inactivate glutathione reductase that accelerates oxidative damage (Turk, 2010). 3-DG also disrupts the glucose metabolism as it inhibits the activities of hepatic enzymes (Turk, 2010). The uremic and diabetic patients have elevated plasma levels of 3-DG (Niwa *et al.*, 1995; Hamada *et al.*, 1997). 3-DG-imidazolone and CML can induce modifications in cytokines and growth factors (Turk, 2010). Therefore, they are believed to contribute to the pathology of diabetic vascular diseases.

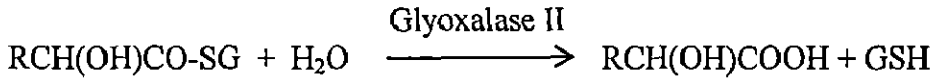
Glyoxal is also an intermediate product of Maillard reaction. Additionally, it is also a by-product of the lipid peroxidation (Turk, 2010). It is a significant precursor of AGEs and has an importance as a glycating agent in physiological systems. Glyoxal-lysine dimer (GOLD) is a lysine-lysine cross linking structure and is a specific glyoxal-derived AGE (Turk, 2010). In diabetic state and aged human beings, the level of GOLD is elevated (Frye *et al.*, 1998; Shamsi and Nagaraj, 1999; Sady *et al.*, 2000). There is a significant increase of plasma glyoxal level in diabetic patients (Lapolla *et al.*, 2003; Han *et al.*, 2007). CEL and its homologue CML is formed by the reaction of MG and glyoxal with proteins, respectively (Ahmed *et al.*, 1997). Therefore, oxidative stress can be indicated by excess amount of MG, CEL and CML in tissues (Baynes and Thorpe, 1999; Vlassara *et al.*, 1994; Ando *et al.*, 1999; Li *et al.*, 1996; Sugimoto *et al.*, 1997). Therefore, there is a significant role of dicarbonyl compounds and oxidative stress in the chemical modification of proteins in aging and disease (Ahmed *et al.*, 1997).

1.2.3.1. Metabolism of dicarbonyl compounds

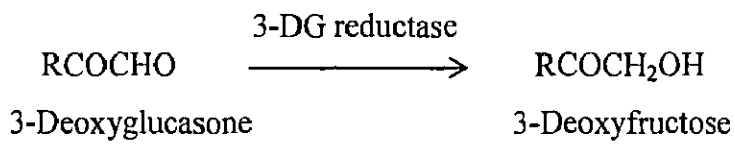
The glyoxylase system is an important metabolic pathway and is found in the cytosol of cells and cellular organelles, particularly mitochondria. It is present in all forms of life (Carrington and Douglas, 1986). The glyoxalase system catalyzes the conversion of MG and glyoxal into D-lactate and glycolate, respectively (Thoranalley, 2003). Two enzymes, glyoxalase I and glyoxalase II complete this catalytic reaction and they work in a sequential manner. Reduced glutathione (GSH) is required for the activity of both enzymes (Carrington and Douglas, 1986).

The reactions are:





Detoxification of 3-DG results in the formation of 3-deoxyfructose and is catalysed by NADPH dependent aldehyde reductase and aldose reductase (Feather, 1995).



The oxidation of 3-DG to 3-deoxygluconate is catalyzed by 2-oxoaldehyde dehydrogenase (Fujji *et al.*, 1995)

1.2.4. Prevention of glycation

Controlling blood sugar level is a very effective and natural method to prevent excessive glycation in diabetes. Basically, glycation can be prevented by natural defence system present inside the body or by inhibitors that may be synthetic or natural.

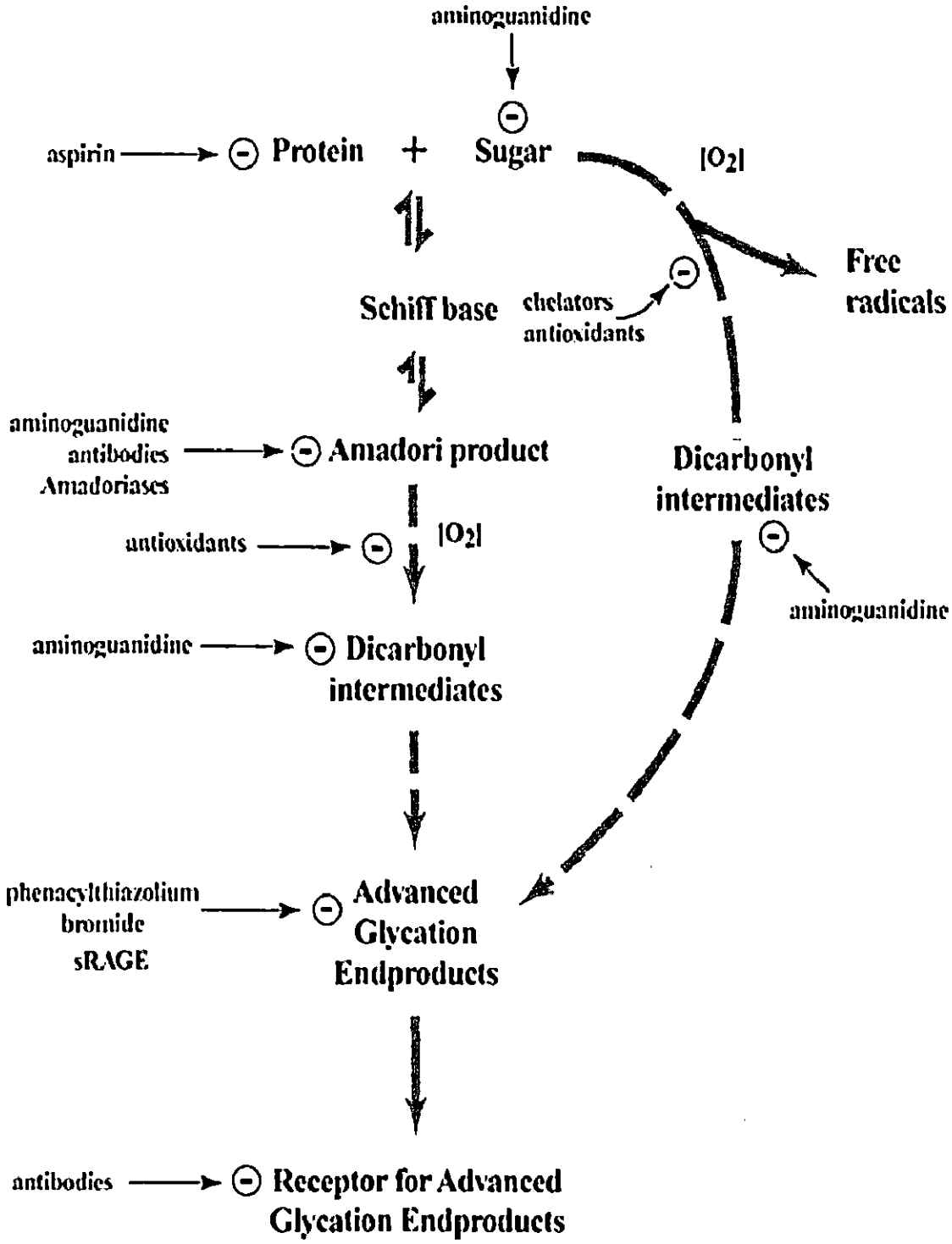
1.2.4.1. Natural defence

Several researchers have proposed the concept of an enzymatic defense against glycation that offers protection against cell damage mediated by glycation. This concept is based on the enzymatic activities that involves the prevention/suppression of glycation adducts formation and catalysis of glycated protein repairing. Glyoxalase system (both I and II), aldehyde reductase, aldose reductase and the liver enzyme, α-ketogluteraldehyde dehydrogenase are considered to be included in these natural enzymatic defence mechanisms against glycation and AGEs accumulation (Thornalley, 1990; Thornalley, 1998). Amadoriosis, an exclusive enzyme found in *Aspergillus*, catalyzes the deglycation of Amadori products (Gerhardinger *et al.*, 1995). Human fructosamine-3-kinase (FN3K) is able to reverse the non-enzymatic glycation at an early stage (Szwergold *et al.*, 2001). Reactive dicarbonyl compounds are detoxified by oxaldehyde dehydrogenase and aldehyde reductase that are NADPH dependent (Boel *et al.*, 1995; Suzuki *et al.*, 1998). During the reduction of peroxide or superoxide, gluthathione (GSH) system work both as antioxidant and coenzyme. Furthermore, GSH has an important role to facilitate the detoxification of MG in glyoxylase pathway (Thornalley, 1998). Numerous plasma amines are present in body that may react with sugar and Amadori carbonyl groups to decrease AGEs. Extracellular ligand-binding domain of the receptor or soluble RAGE can be used to block RAGE and this is shown to enhance wound closure in genetically diabetic mice (Goova *et al.*, 2001).

1.2.4.2. Antiglycating compounds

Several types of antiglycating agents have been described (Taha *et al.*, 2014). These can interefere with different potential sites to inhibit glycation and AGE formation (Fig. 8). Some may have the ability to compete for the amino groups on the protein (Harding and Ganea, 2006; Brownsen and Hipkiss, 2000). Other can

Fig. 8. Potential sites where pharmacological compounds can act to inhibit protein glycation and AGE-mediated damage (Ahmed *et al.*, 2005).


aminoguanidine
aspirin
Protein
+
Sugar
|O2|
Free radicals
Schiff base
chelators
antioxidants
aminoguanidine
antibodies
Amadoriases
Amadori product
Dicarbonyl intermediates
antioxidants
|O2|
aminoguanidine
aminoguanidine
Dicarbonyl intermediates
phenacylthiazolium bromide
sRAGE
Advanced Glycation Endproducts
antibodies
Receptor for Advanced Glycation Endproducts

directly bind to the protein or can bind the glycation intermediates to stop the progression up to AGE formation stage (Harding and Ganea, 2006). Otherwise, they may have the property to eliminate the open chain form of glycating sugars (Harding and Ganea, 2006). Furthermore, several probable AGE inhibitors have been proposed (Rahbar and Figarola, 2003; Rahbar *et al.*, 2000). Various inhibitors have been developed and some of them are in advanced clinical studies/trials (Williams, 2004; Giannoukakis, 2005; Thomas *et al.*, 2005). The concept of inhibitory mechanism is primarily concentrated on blocking of the sugar attachment to proteins, attenuating glycooxidation and oxidative stress through trapping or scavenging some glycation intermediates including ROS, reactive nitrogen species (RNS) and dicarbonyls as well as breakage of AGEs crosslinks (Reddy and Beyaz, 2006).

Primarily, inhibitors can be divided into two groups: synthetic and natural inhibitors.

1.2.4.2.1. Synthetic inhibitors

(1) Inhibitors interfering with sugar attachment with proteins

It has been found that only a few of the synthetic inhibitors have the property to interfere with the initial attachment of reducing sugars to the amino groups of proteins. For example, aspirin inhibits the glycation process by acetylating free amino groups of proteins therefore it can block the attachment of reducing sugars with amino groups (Crompton *et al.*, 1985; Rao *et al.*, 1985). An anti-inflammatory drug, diclofenac can make a covalent interaction with proteins and thus, can block the attachment of sugars with proteins. It has been shown to block at least one of the major glycation site of human serum albumin (Van-Boekel *et al.*, 1992). Inositol is a synthetic and potent antiglycating agent because glucose can be scavenged out by inositol, and it was found that the glycation process decreased by 57-67 % in human eye lens protein in the presence of inositol (Ramakrishnan *et al.*, 1999). Arginine and arginine-lysine can prevent the alterations of rat tail tendon that are induced by glycation because of competitive attachment of these amino acids to glucose (Mendez and Leal, 2004). Metformin is a well-known blood sugar lowering agent and has been reported to have moderate inhibitory effects on early stage of glycation (Rahbar and Figarola, 2003). Pioglitazone and pentoxifylline are other synthetic drugs that exert the same inhibitory effect on early stage of glycation (Rahbar *et al.*, 2000).

(2) Inhibitors inhibiting the late stage of glycation

Some synthetic inhibitors have the ability to scavenge both reactive carbonyls and reactive free radicals formed during glycation or can block the formation of Amadori products. Aminoguanidine (AG) and pyridoxamine are potent carbonyl and free radical scavengers and have been widely studied to investigate their AGE inhibiting property. AG was the first AGE inhibitor to be studied both *in vitro* and *in vivo* (Brownlee *et al.*, 1986). AG has a significant potential to react with dicarbonyl intermediates formed during glycation (Brownlee, 1986; Corbett *et al.*, 1992). Thus, AG helps in prevention of progression of diabetic complications (Thornalley, 2003). Previous studies have provided the evidences that AG is not only a potent AGE inhibitor, but also works in prevention of diabetic complications that include nephropathy, neuropathy and vasculopathy (Thornalley, 2003). Pyridoxamine and thiamine pyrophosphate are potential dicarbonyl scavenger and have a strong inhibitory effect on AGE formation. Pyridoxamine offers more protection against AGE formation (Metz *et al.*, 2003; Voziyan *et al.*, 2002; Khalifah *et al.*, 1999) as compared to AG (Booth *et al.*, 1996) by trapping dicarbonyl compounds (Ahmed *et al.*, 2005; Voziyan *et al.*, 2002). Thiamine pyrophosphate has also comparable inhibitory effect on AGEs formation (Booth *et al.*, 1997).

Buformin (Kiho *et al.*, 2005) and carnosine (Hipkiss *et al.*, 1994; Yan and Harding, 2005) can prevent *in vitro* protein glycation and cross linking.

(3) Inhibitors with radical scavenging properties

Some compounds are reported to retard or suppress AGE formation because of their possible radical scavenging properties. Calcium antagonists (Sobal *et al.*, 2001), amlodipine (Akira *et al.*, 2006), kinetin (Verbeke *et al.*, 2000) and quinine (Jung *et al.*, 2005) are able to retard or supress AGE formation possibly due to radical scavenging abilities.

(4) Inhibitors with property to inhibit Amadori product formation

Some antiglycating compounds have the ability to inhibit the formation of Amadori products. Tenilsetam is able to attach with sugar-derived moieties of glycated proteins (Munch *et al.*, 1994). Thus, reactive sites are blocked and this stops the further polymerization reactions. Some researchers have also

reported the inhibition of formation of Amadori products and reduction in the level of AGEs by pencillamine (Stevens, 1995). Ethanol can be metabolized into acetaldehyde *in vivo* which forms a stable complex with Amadori products, thus ethanol may exert inhibitory effect on AGE formation (Al-Abed *et al.*, 1999).

(5) Cross-link breakers

Cross-linking between AGEs and proteins is the reason behind the stiffening of arteries and cardiovascular damages (Zieman *et al.*, 2005). Hence, breakage of AGE cross-links is a very good method to prevent diabetic complications caused by cross-linking. The concept of AGE breakers suggests that they can release albumin from preformed AGE-albumin-collagen complexes and can be able to dissociate the immunoglobulin adducts from red cells of diabetic rats (Nagai *et al.*, 2012). Another view implicates the involvement of AGE breakers in the prevention of cross-linking and/or reversing of the cross-links once they are formed (Nagai *et al.*, 2012; Susic, 2007). One more way by which AGE breakers can work is metal chelation (Price *et al.*, 2001).

N-phenacylthiazolium bromide (PTB) was the first cross-link breaker reported (Vasan *et al.*, 1996). Alagebrium (ALT-711) is a small synthetic compound and is able to decrease cardiovascular stiffening (Asif *et al.*, 2000) and drug retarded nephropathy (Forbes *et al.*, 2003) in diabetic rats. Furthermore, TRC4186 is a pyridinium analogs that are able to break AGE cross-links (Chandra *et al.*, 2009).

(6) Other miscellaneous synthetic inhibitors

Some anti-inflammatory drugs including acetylsalicylic acid, ibuprofen, indomethacin and diclofenac act as antiglycating agents because they inhibit oxidative stress (Shastri *et al.*, 1998; Caballero *et al.*, 2000). Specific iron chelators like desferoxamine was also helpful in treating diabetes (Liu *et al.*, 2009). Aldose reductase inhibitors (ARIs) can block excessive glucose metabolism. Eplarestat is an ARI and it brings about the lowering of the level of fructose-3-phosphate in diabetic patients (Hamada *et al.*, 1995) and also lowers down imidazolone and CML (AGEs) due to decreased peroxidation of lipids (Tsukushi *et al.*, 1999).

Angiotensin II Receptor Blocker (ARB) and Angiotensin converting Enzyme Inhibitors (ACEI) also have inhibitory effects on AGEs. The possible mechanisms of action may include chelating of metal ions, scavenging of free radicals, trapping of carbonyl compounds and/or inhibition of carbonyl compound production (Miyata *et al.*, 2002).

1.2.4.2.2. Natural inhibitors

Although synthetic compounds are strong antiglycating agent or strong inhibitors of AGE formation, they might exert severe adverse effects. For example, the clinical trials of AG were terminated because of safety concerns because AG was found to have many adverse effects like gastrointestinal disturbances, rare vasculitis, anaemia and flu-like symptoms (Freedman *et al.* 1999). Its interference with vitamin B6 metabolism is another major limitation (Miyata *et al.*, 2002). Metformin, another synthetic antiglycating compound exerts several adverse effects including nausea and diarrhoea (Kavishankar *et al.*, 2011). Therefore, recently much interest has been developed in the search of natural phytochemicals from plants that effectively inhibit glycation and have fewer side effects (Coman *et al.*, 2012). Naturally occurring phytochemicals/products have been found to be relatively safe for human consumption as compared to synthetic compounds and are relatively non-toxic, inexpensive and are available in an ingestible form. A large number of plants and natural biomolecules have been discussed in literature for their antidiabetic effects (Coman *et al.*, 2012; Bailey and Day, 1989; Soumyanath, 2006). Some plant extracts, fractions and compounds have been tested for antiglycating activities (Pang *et al.*, 2011) However, the mechanism is often not completely understood.

It is well established that glycation and AGEs formation are accompanied and accelerated by oxidative stress, therefore antioxidant compounds may be promising agents for the prevention of glycation and AGE formation. Polyphenolic compounds especially flavonoids have received most attention with regard to their antidiabetic properties (Soumyanath, 2006). Anthocyanins are flavonoids with high antioxidant capacity. Many plants have been used by the local Indian tribes for antidiabetic therapeutics since a long time, but only a few of them have been scientifically studied e.g. *Flemingia macrophylla, Potentilla fulgens* L., *Albizzia lebbek, Curcuma amada, Gymnoptela cochinchinesis* and *Ixeris gracilis* DC (Syiem and Warjri, 2011).

Methylcaffeate from *Solanum torvum* is found to have a hypoglycemic effect (Gandhi *et al.*, 2011). Aqueous/ethanolic extracts of *Allium cepa* (skin), *Illicium religiosum* (bark and wood), *Fagopyrum esculentum* (hull), *Origanum officinalis* (leaf) are proved to have effective antiglycating and antioxidant properties (Kim and Kim, 2003). The results also showed that their antiglycating activities significantly correspond to their antioxidative capacities (Kim and Kim, 2003). Aged garlic extract is a potential inhibitor of AGEs with potent antioxidant activities (Ahmad and Ahmad, 2006). One major component of garlic extract, allicin is a sulfur-containing compound and has been shown to have significant hypoglycemic activity (Sheela and Augusti, 1992). The age-related increase in collagen cross-linking and fluorescent products in C57BL/6 mice can be decreased by green tea extract (Rutter *et al.*, 2003). The activity of green tea extract is mainly due to tannin components, which provide protection against oxidation and glycation (Nakagawa *et al.*, 2002). Aloe vera (*A. vera*) also has antidiabetic and lipid-lowering properties, since oral administration of its extract significantly reduced fasting blood glucose level and improved lipid profile status in streptozotocin-induced diabetic rats (Rajasekaran *et al.*, 2006). The methanol extract of *Salacia chinensis* stems provides strong inhibition against the formation of Amadori compounds and AGEs in addition to its anti-hyperglycemic action (Yoshikawa *et al.*, 2003). The seeds of *Acacia arabica* induced hypoglycemic effect in mice by initiating the release of insulin from pancreatic beta cells (Yoshikawa *et al.*, 2003). The aqueous extract of *Aegle marmelos* leaves reduces blood sugar and urea and serum cholesterol in diabetic rats as compared to the control (Karunanayake *et al.*, 1984).

Azadirachta indica is widely distributed throughout India. The leaf extracts of this plant have anti-hyperglycemic effect in diabetic rats (Chattopadhayay, 1999). *Ocimum santalum* is considered to be a sacred plant in Indian culture. Its aqueous extract significantly reduces the blood sugar level in diabetic rats (Vats *et al.*, 2002). The aqueous extract of *Mangifera indica* also proved to have hypoglycemic activity which may be due to the reduction of the intestinal absorption of glucose (Aderibigbe *et al.*, 1999). *Momordica charanta* is considered to be very popular antidiabetic and antihyperglycemic vegetable in India as well as in other Asian countries. The hypoglycemic effect of extracts of its fruit pulp, seed, leaves and whole plant have been proved in various animal models (Khanna *et al.*, 1981). *Phyllanthus amarus* is a

herb and is used by the local peoples of south India in treating diabetes. Potent antioxidant activity has been found in the methanolic extract of *P. amarus*. In diabetic rats, its extract reduced the blood sugar level (Raphael *et al.*, 2002). In addition, several studies have reported the AGE formation and crosslinking inhibiting potential of curcumin in diabetic rats (Rahbar and Figarola, 2003).

Quercetin is a flavonoid and belongs to subclass flavonols (Hartog *et al.*, 1993) (Fig. 9) and is widely distributed in many plants: flowers, leaves, and fruits. Quercetin possesses strong anti-diabetic activity (Eid *et al.*, 2015). It has the ability to trap MG and glyoxal and thus, can inhibit AGEs formation (Li *et al.*, 2014). Quercetin offers protection against lipid peroxidation and also provides antioxidant effects in diabetes (Groot and Rauen, 1998). It is a strong antioxidant (Afanas'ev *et al.*, 1989).

1.3. Natural products used in this study

1.3.1. Thymoquinone (TQ)

TQ, 2-Isopropyl-5-methyl-1,4-benzoquinone, is one of the most active ingredients of *Nigella sativa* seeds (Rahmani *et al.*, 2014) (Fig. 10 A and B). *Nigella sativa* is commonly known as black seed or black cumin and is an annual flowering plant. It is a member of Ranunculaceae (Nahas and Moher, 2009) and is native to southwest Asia. This plant is widely cultivated and distributed in southern Europe, northern Africa and Asia Minor (Salem, 2005). It is considered to be a medicine with great healing power in the Islamic world. There is a belief that Prophet Muhammad (PBUH) said "Use Black seed regularly, since it is a cure for every disease except death" (Althaus *et al.*, 1978). The black seeds have been used for the treatment of many disease conditions including bronchial asthma, headache, dysentery, infections, obesity, back pain, hypertension, gastrointestinal problems (Salem, 2005). They have been used for centuries in the traditional system of medicine for example, Ayurveda, Unani, Arabic and Chinese medicine. Pharmacologically important activities of the black seed include anti-asthma, anti-diarrhoea and anti-dyslipidaemia, antiinflammatory, analgesic, antipyretic, antimicrobial and antineoplastic activities (Ali and Blunden, 2003). The oil is found to decrease the blood pressure and helps to increase the respiration (Ali and Blunden, 2003). Furthermore, the seeds have been reported to have potent antioxidant properties both *in vitro* and *in vivo*, anti-diabetic, anti-inflammatory, immunomodulatory, antimicrobial, anti-viral, anti-helminthic,

Fig. 9. Chemical structure of quercetin

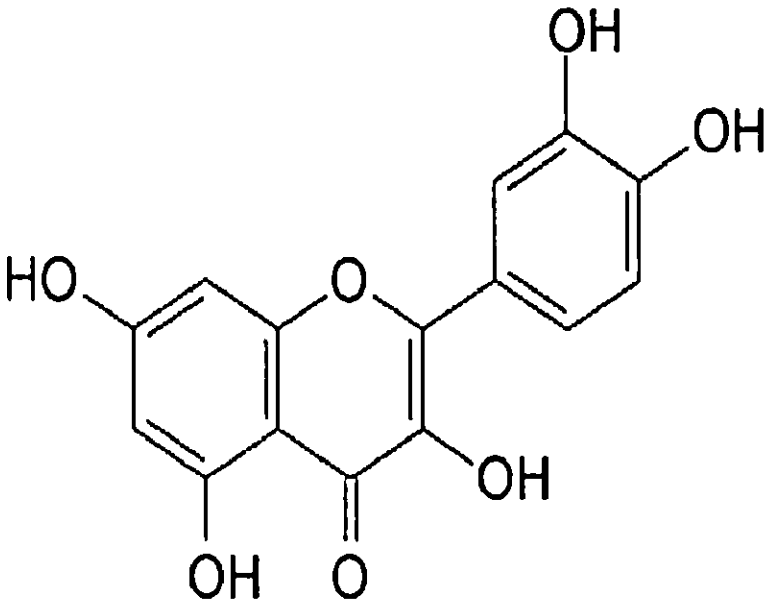
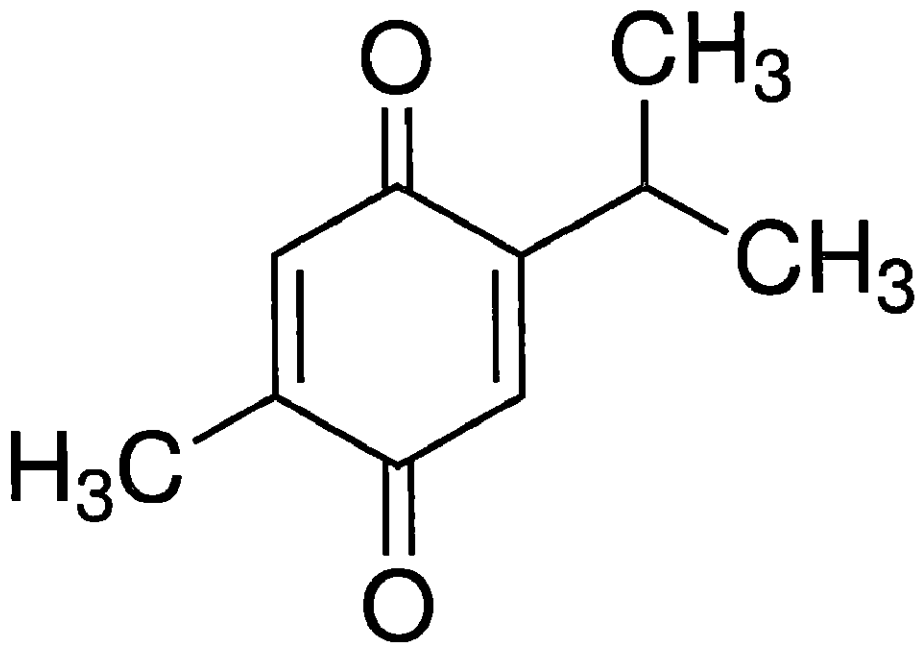

OH
OH
HO
O
OH
OH
O

Fig. 10A. Black cumin seeds

Fig. 10B. Chemical structure of thymoquinone

B



anti-bacterial, anti-tumour, antihypertensive, galactagogue and insect repelling effects (Salem, 2005, Riaz *et al.*, 1996; Siddiqui and Sharma, 1996; Worthen *et al.*, 1998). Most of the pharmacological properties of black seeds are devoted by quinone constituents, and TQ is the prime active component of the volatile black seed oil (Aboutabl *et al.*, 1986). TQ is a potent O_2^- scavenger and is found to have comparable scavenging power as SOD, and it can alter the redox state by scavenging ROS including O_2^-, $HO^·$, H_2O_2, $ONO_2^·$ free radicals through modulation of hepatic and extrahepatic antioxidant enzymes (Mansour *et al.*, 2002; Hamdy and Taha, 2009). Furthermore, TQ has been found to offer protection against the kidney damage induced by ifosfamide, mercuric chloride, cisplatin and doxorubicin by checking renal GSH depletion and antilipid peroxidation product accumulation, hence improving renal functioning (Fouda *et al.*, 2008).

The antioxidant properties of TQ and its metabolite dihydrothymoquinone (DHTQ) may play at least in part for the pharmacological action of TQ. For example, cellular damage caused by oxidative stress can be clinically prevented by TQ (Sankaranarayanan and Pari, 2011). TQ acts as a free radical scavenger, and also preserves the activity of various anti-oxidant enzymes such as catalase, glutathione peroxidase and glutathione-S-transferase (Sankaranarayanan and Pari, 2011; Syed-Ahmad *et al.*, 2010; Woo *et al.*, 2012). The anticancer effect(s) of TQ are mediated through different modes of action, including antiproliferation, apoptosis, induction, cell cycle arrest, ROS generation and antimetastasis/anti-angiogenesis (Woo *et al.*, 2012). The combination of TQ and conventional chemotherapeutic drugs produce greater therapeutic effects and also reduces the toxicity of the latter (Al-Majed *et al.*, 2006).

1.3.2. *A. vera* and aloin

A. vera is *Aloe barbadensis* and is a member of the liliaceae (Asphodelaceae) family (Surjushe *et al.*, 2008) (Fig. 11A). It is a perennial, shrubby, succulent, xerophytic, pea-green coloured plant that grows in arid and subtropical climate. It contains several, almost 75 biologically active substances that can be grouped as vitamins, minerals, amino acids, enzymes, anthraquinone, saccharides, sugars, lignin, salicylic acids and fatty acids (Atherton *et al.*, 1998; Shelton, 1991). *A. vera* has been used as a popular folk medicine throughout history. At least six antiseptic agents

including lupeol, salicylic acid, urea nitrogen, cinnamonic acid, phenols and sulphur can be obtained from this plant that are capable of killing various pathogens such as bacteria, mold and viruses (Rajeshwari *et al.*, 2012). The thick fleshy leaves of *Aloe* plants contain pulp which is used in the food, pharmaceutical, cosmetic and toiletry industries (Nejatzadeh-Barandozi, 2013). *A. vera* has been shown to have several benefits including healing properties (Chithra *et al.*, 1998; Heggers *et al.*, 1996), protective effect against radiation damage to the skin (Roberts and Travis, 1995; Sato *et al.*, 1990) and anti-inflammatory action (Hutter *et al.*, 1996). The benefits of *A. vera* that are not supported by experimental/clinical data are treatment of acne, anemia, haemorrhoids, glaucoma, petit ulcer, tuberculosis and blindness (Wani *et al.*, 2010). Many studies have reported the antioxidant, anti-inflammatory and antibacterial activities of *A. vera* (Lopez *et al.*, 2013; Nejatzadeh-Barandozi, 2013). Anti-cancer effects of a compound of *A. vera* leaves, aloe-emodin has also been reported (Lin *et al.*, 2010). *A. vera* also has antidiabetic and lipid-lowering properties since oral administration of the extract significantly reduced fasting blood glucose level and improved lipid profile status in streptozotocin-induced diabetic rats (Moniruzzaman *et al.*, 2012; Rajasekaran *et al.*, 2006). The phytochemicals present in *A. vera* are tannins, phlobatannins, saponins, flavonoids, steroids, terpenoids and cardiac glycosides anthraquinones, which have medicinal value (Sathyaprabha *et al.*, 2010). Phenolic compounds are the second major substances in *A. vera*.

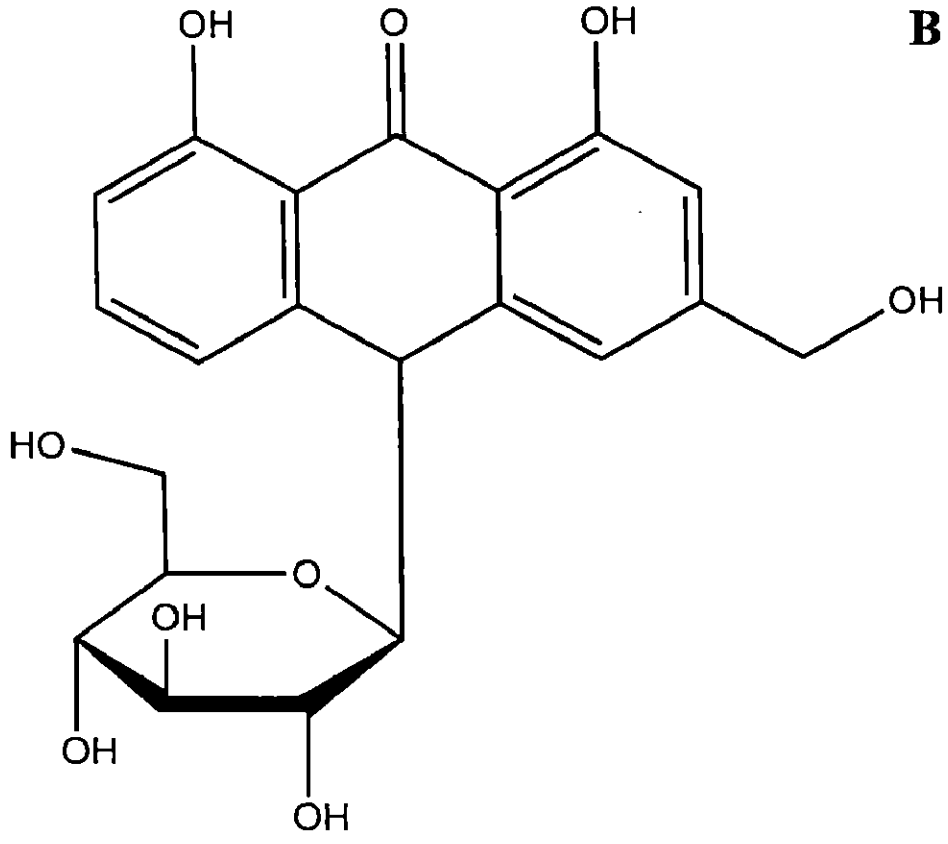
The main active constituent in *A. vera* extract is aloin, an anthraquinone heteroside (Zahn *et al.*, 2008) (Fig. 11B). Its IUPAC name is 8-Dihydroxy-10-(β-D-glucopyranosyl)-3- hydroxymethyl)-9(10H)-anthracenone (Femenia *et al.*, 1999). The antioxidant activity of aloin has been demonstrated (Esmat *et al.*, 2012). Anti-tumour activity of aloin has also been reported (Esmat *et al.*, 2005; Fahim *et al.*, 1997). Aloin is a potential drug candidate for cancer therapy because it can block the activation of STAT3 which causes the inhibition of tumor angiogenesis and growth (Qin-Pan *et al.*, 2013). It was shown to induce apoptosis in Jurkat cells (Buenz, 2008). Aloin has a significant cytotoxic effect in human epithelial type breast and ovarian tumor cell lines (Esmat *et al.* 2006) and in human uterine carcinoma HeLaS3 cells (Niciforovic *et al.* 2007) by inducing S-phase cell cycle arrest and apoptosis. It also has chemopreventive effects against 1,2-dimethylhydrazine-induced preneoplastic lesions in the colon of Wistar rats (Hamiza *et al.*, 2014).

Fig. 11A. ***Aloe vera*** **plant**

Fig. 11B. Chemical structure of aloin

A

B



The antibacterial potential of *Aloe* depends on the concentration of aloin, which because of its glycosides, can easily enter the cells (Tian *et al.*, 2003). Aloin has been found to inactivate several enveloped viruses that include *Herpes simplex*, *Varicella zoster* and *Influenza* viruses (Sydiskis *et al.*, 1991). Aloin and its derivatives have the antitrypanosomal potential (Tewabe *et al.*, 2014). Aloin also contributes to the radical scavenging activity of *A. vera* (Lucini *et al.*, 2014). It has also been shown to have excellent laxative effect (Beppu *et al.*, 2003; Park *et al.*, 2009). Aloin has a protective effect against chronic alcoholic liver injury via attenuating lipid accumulation, oxidative stress and inflammation in mice (Cui *et al.*, 2014).

1.3.3. Ellagic acid (EA)

EA is a thermostable polyphenolic molecule (Fig. 12), which is a dimeric derivative of gallic acid, and is found in a wide variety of fruits and nuts that include raspberries, strawberries, walnuts, grapes, black currants (Zafrilla *et al.*, 2001; Ancos *et al.*, 2000), either in its free form, as glycosides, or as in bound form such as ellagitannins (Amakura *et al.*, 2000). A high content of EA is found in blackberries, strawberries, and raspberries (Rommel and Wrolstad, 1993). Furthermore, 88% of the total phenolic content analyzed in raspberry fruits is due to EA (Hakkinen *et al.*, 1999).

EA has a strong antioxidant property which is due to its phenolic groups (Solon *et al.*, 2000; Festa *et al.*, 2001). It can trap free radicals and hence reduces oxidative stress and this property may be very useful in the treatment of several diseases. EA has been found to have several beneficial activities including antimutagenic, antimicrobial and HIV inhibiting potential (Feldman *et al.*, 1999; Akeyama *et al.*, 2001; Vettom and Shetty, 2003; Ruibal *et al.*, 2003), and chemoprotective activity (Ahn *et al.*, 1996).

EA has been shown to maintain the glucose homeostasis in streptozotocin induced diabetic rats (Malini *et al.*, 2011). EA and its derivatives can inhibit sorbitol accumulation (Ueda *et al.*, 2004). Diseases of the kidney, eye, heart and joints caused by high blood glucose levels could be prevented by EA (Ventura-Sobrevella *et al.*, 2009). It increases the activity of insulin and brings about the reduction of inflammation as well as oxidative stress (Seeram *et al.*, 2005). EA was shown to

exhibit cytotoxic and antiproliferative activities in lung, colon, breast and prostate cancers (Losso *et al.*, 2004). It is postulated that the metastatic factors are inhibited by EA which causes the apoptosis of cancer cells (Losso *et al.*, 2004). Furthermore, EA was observed to have more than one mechanism of action to work as antitumor agent, either scavenging oxygen species produced by H_2O_2 treatment or/and protecting DNA double helix from injury by alkylating agents (Cozzi *et al.*, 1995). Some dietary supplements including EA inhibit mutagenesis and hence have anticarcinogenic potential (Hayatsu *et al.*, 1988). EA has been shown to be cytotoxic to oral carcinoma cells but not to normal cells (Weisburg *et al.*, 2013). It has been reported to induce antiproliferation, cell cycle arrest and death in several human cancerous cells (Li *et al.*, 2005; Losso *et al.*, 2004; Mertens-Talcott *et al.*, 2005; Narayanan *et al.*, 1999).

EA is a good antiviral as well as antimicrobial agent (Akiyama *et al.*, 2001). It suppresses the growth of pathogens in humans probably by coupling with the proteins of the bacterial cell wall, including *Bacillus, Staphylococcus* and *Salmonella* (Akiyama *et al.*, 2001). Some EA derivatives isolated from the bark of *Elaecarpus parvifolius* have potent antiparasitic activity against *Babesia gibsoni* (ElKhateeb *et al.*, 2005).

1.3.4. Alliin

Garlic (*Allium sativum* L.) is a member of *Alliaceae* family. It has been used in different countries in diet and also as a medicinal agent over centuries. It is widely taken in raw form as well as like a medicinal agent against several diseases (Jastrzebski *et al.*, 2007; Gorinstein *et al.*, 2006). Garlic has many health beneficial properties because of its ability to protect against several diseases. Garlic can be used in prevention as well as therapy of several diseases including cardiovascular diseases, atherosclerosis, hyperlipidemia, thrombosis, hypertension and diabetes (Agarwal, 1996). It has been shown to regulate plasma lipid levels (Steiner and Li, 2001) and increases the anticoagulant activity of plasma (Apitz-Castro *et al.*, 1992; Ackermann *et al.*, 2001). It is used as a remedy in many diseases due to its prophylactic and therapeutic action (Bhagyalakshmi *et al.*, 2005; Kabasakal, *et al.*, 2005; Lawson and Gardner, 2005). It is found to be effective against a wide number of microorganisms, hence it is considered to be a natural antibiotic (Adetumbi and Lau, 1983). The

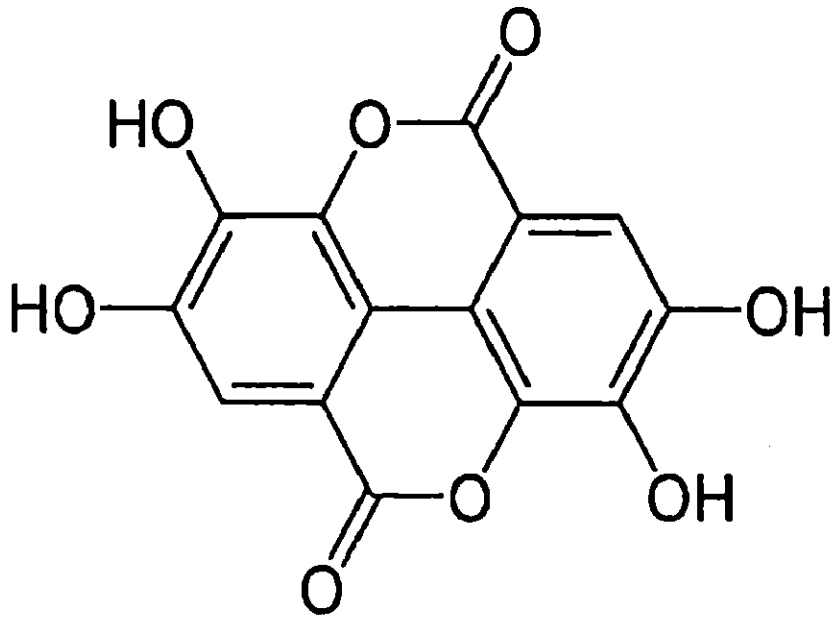
minimum inhibitory concentration of aqueous garlic extract against isolate of multidrug resistant bacteria was found to be 5µl/ml (Chowdhury *et al.*, 1991). Garlic has been shown to reduce the blood sugar level in streptozotocin treated rat models (Patumraj *et al.*, 2000). Furthermore, garlic was also able to reduce the blood glucose level in rat models having diabetes mellitus caused by alloxan treatment (Augusti and Sheela, 1996; Kumar and Reddy, 1999). These results strongly indicate that garlic has antidiabetic properties. The ability of garlic to inhibit cancer has also been reported (Dausch and Nixon, 1990; Dorant *et al.*, 1993; Amagase and Milner, 1993; Fleischauer and Arab, 2001). Garlic is a rich sources of sulphur containing organic compounds such as alk(en)yl cysteine sulphoxides (CSOs), alliin, isoalliin and methiin that provide aroma, flavour and health beneficial properties to it.

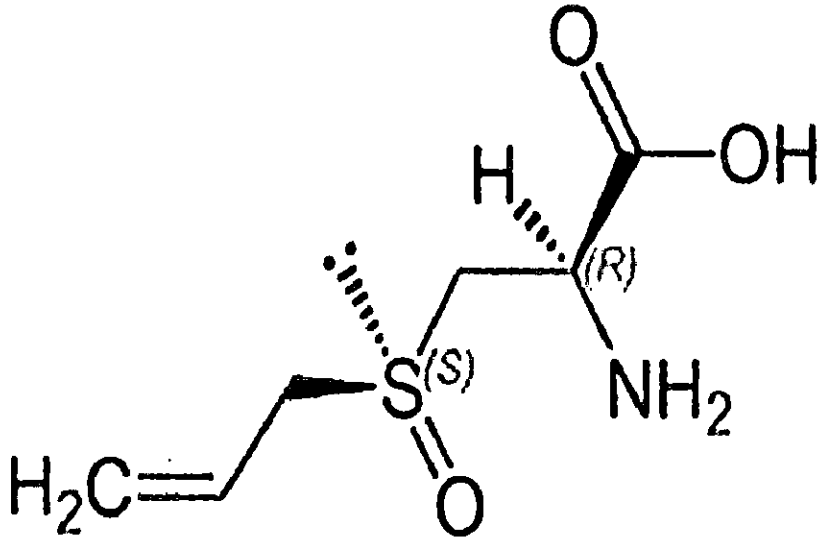
Alliin (S-allyl-L-cysteine sulfoxide) is a derivative of an amino acid cysteine, and is an organosulfur compound that contains an allyl group, a sulfoxide group and an amino acid cysteine (Fig. 13). Alliin has been reported to exhibit several valuable health beneficial properties including antidiabetic (Sheela and Augusti, 1992), anticholesterolemic (Sheela and Augusti, 1995) and anticarcinogenic (Le Bon *et al.*, 2003). Alliin was shown to have significant antidiabetic effects in alloxan treated diabetic rats (Sheela and Augusti, 1992). It ameliorated the diabetic condition up to almost the same point as gibenclamide and insulin did. Furthermore, alliin stimulated isolated B cells to secrete insulin *in vitro* (Augusti and Sheela, 1996). It has the potential to scavenge the hydroxyl radicals and this potential is considered to be responsible for its biological activities (Lachmann *et al.*, 1994). Cardioprotective effect of alliin has also been reported in myocardial infarcted male Wistar rats (Sangeetha and Darlin-Quine, 2007; Sangeetha and Darlin-Quine, 2006a; Sangeetha and Darlin-Quine, 2006b). Garlic and its alliin exhibit anti-inflammatory and antioxidant properties and hence have the potential to inhibit the atherosclerosis (Hui *et al.*, 2010). The extract of garlic and also alliin has been found to be potent antioxidants (Kourounakis and Rekka, 1991). Alliin scavenges O_2^- as well as OH^- (Chung, 2006).

On crushing of garlic, the enzyme alliinase present in garlic converts alliin to allicin (diallyl disulfide-oxide) (Amagase *et al.*, 2001; Lawson and Wang, 2001). Allicin also exhibits many important properties that include antifungal and antibacterial properties (Ilic *et al.*, 2010; Khodavandi *et al.*, 2010; Ankri and Mirelman, 1999).

Fig. 12. Chemical structure of ellagic acid

Fig. 13. Chemical structure of alliin


HO
O
O
HO
OH
O
OH
O


O
OH
H
(R)
S(S)
NH2
H2C
O

1.4. The present study

Diabetes has become the most common metabolic disease worldwide. Hyperglycemia has a key role in the diseases associated with diabetic complications. Glycation of proteins plays an important role in the development of physiological and pathophysiological processes, such as aging, diabetes, atherosclerosis, neurodegenerative diseases, vascular diseases and chronic renal failure (Brownlee, 1995). SOD, the important antioxidant enzyme in the body which counters the deleterious effects of ROS, also gets inactivated by glycation (Jabeen *et al.*, 2006). Exposure of SOD to glucose results in its deactivation following by site-specific and random fragmentation (Jabeen *et al.*, 2007). The damage caused by oxidative stress is expected to be exacerbated if the antioxidant enzymes themselves are inactivated by glycation. Elevated levels of MG have been reported to adversely affect SODs particularly against $O_2^{\cdot-}$. Exposure of SOD to MG has been shown to cause its covalent cross-linking associated with loss of enzymatic activity (Kang, 2003).

Current antidiabetic therapy is based on synthetic drugs that very often have side effects (Codario, 2005). Alternative medicines and natural therapies have stimulated new interest of research to find for more efficacious agents with lesser side effects. Furthermore, much interest has been developed in the search of natural phytochemicals from plants that effectively inhibit glycation and have fewer side effects (Coman *et al.*, 2012). Naturally occurring phytochemicals/products have been found to be relatively safe for human consumption as compared to synthetic compounds, and are relatively non-toxic, inexpensive and are available in an ingestible form. A large number of plants and natural biomolecules have been discussed in the literature for their antidiabetic effects (Coman *et al.*, 2012; Bailey and Day, 1989; Soumyanath, 2006). Some plant extracts and compounds have been tested for their antiglycating potential in the past few years (Peng *et al.*, 2011). The mechanism is most often not completely understood.

A large number of hypoglycaemic compounds have antioxidant properties. Throughout history black cumin seeds, have been one of the most revered medicinal seeds. TQ, an active principle component of the volatile oil of these seeds, possess anti-diabetic, anti-oxidant, hepatoprotective, neuroprotective, nephroprotective, anti-tumor and anti-mutagenic pharmacological activities (El-Mahmoudy *et al.*, 2005;

Erkan *et al.*, 2008; Daba and Abdelrahman, 1998; Al-Majed *et al.*, 2006; Fouda *et al.*, 2008; Gali-Muhtasib *et al.*, 2004; Badary *et al.*, 2003). Garlic and its preparations have been traditionally used in food and medicines throughout India. Alliin, the most abundant sulfur compound in garlic, has been reported to exhibit several valuable health beneficial properties including antidiabetic, anticholesterolemic, anticarcinogenic effects (Sheela and Augusti, 1992; Sheela and Augusti, 1995; Le Bon *et al.*, 2003). EA is found in a wide variety of fruits and nuts (Zafrilla *et al.*, 2001; Ancos *et al.*, 2000) and has been found to have several beneficial properties including antimutagenic, antimicrobial, antioxidant, HIV inhibiting potential and chemoprotective, antidiabetic and antitumor activity (Ahn *et al.*, 1996; Losso *et al.* in 2004; Haraguchi *et al.*, 1998; Ueda *et a.l*, 2004). The main active constituent of *A. vera* extract, aloin has antioxidant, anti-inflammatory, antitrypnosomal, antibacterial potential, antiviral and antitumor properties (Esmat *et al.*, 2012; Tian *et al.*, 2003; Esmat *et al.*, 2005; Fahim *et al.*, 1997; Tabolacci *et al.*, 2013; Niciforovic *et al.*, 2007).

Therefore as discussed above, the natural products TQ, *A. vera*, and its component aloin, EA and alliin have antidiabetic activities. The objective of this study was to determine the antiglycating potential of these natural products. The glycation of the important antioxidant enzyme, SOD by glucose or MG and its protection by the above natural products have been studied by activity, SDS-PAGE, ELISA, absorbance, fluorescence and far-UV CD measurements.

Materials and Methods

2. Materials

The chemicals used for this study were obtained from various sources as detailed below.

Chemical	Source
Alliin, Aloin, Bicinchonic acid (BCA), Bovine serum albumin (BSA), Bromophenol Blue, Coomassie blue R-250, Cu,Zn-SOD from Bovine erythrocytes, Ellagic acid, Freund's complete adjuvant, Methylglyoxal [40% aqueous solution], Nitro blue tetrazolium (NBT), Nicotinamide adenine dinucleotide (Reduced) [NADH], O-Phenylenediamine (OPD), Phenazine methosufate (PMS), Thioflavin-T (ThT), N,N, N',N' Tetramethylene-diamine (TEMED), Thymoquinone	Sigma Aldrich, U.S.A.
Acetic acid, Copper sulphate, Dimethyl sulfoxide, Glucose, Glycerol, Methanol, Sodium dodecyl sulphate (SDS), Tris hydroxylmethyla-minomethane (Tris)	Qualigens fine chemicals, India
Acetone, Acrylamide, Ammonium persulphate (APS), Hydrochloric acid, β-Mercaptoethanol, N,N'-Methylene bisacrylamide, Sodium dihydrogen phosphate	Sisco Research Lab, India
Freund's incomplete adjuvant, Horseradish peroxide (HRP) conjugated goat anti-rabbit IgG, Molecular weight protein markers (Broad range)	Genei Pvt. Ltd. Bangaluru, India
Disodium hydrogen phosphate, Glycine	Himedia
Sodium bicarbonate, Sodium carbonate, Sodium chloride	Thermo Fischer Scientific, India

3. Methods

3.1. Measurement of SOD concentration and activity

Commercial SOD gave a single band in the SDS-PAGE and was hence used without purification in the experiments. The concentration of SOD was routinely determined by the BCA method using BSA as the standard (Smith *et al.*, 1985). SOD stock (1 mg/ml) was made in 20 mM sodium phosphate buffer, pH 7.4 and stored at -20°C for future use. The activity of SOD was determined spectrophotometrically by employing PMS–NADH–NBT system (Nishikimi *et al.*, 1972). The reaction mixture consisted of 20 mM sodium phosphate buffer (pH 8.2), PMS (1.9 µM), NBT (184 µM) and NADH (205 µM). All solutions were prepared at ambient temperature in glass vessels under subdued fluorescent room light. For assaying, SOD enzyme was pipette into a cuvette at room temperature (25°C) containing freshly prepared NBT and NADH. The reaction was initiated with the addition of freshly prepared PMS and the absorbance at 560 nm was continuously monitored as an index of NBT reduction using a single beam Shimadzu spectrophotometer. Reagent control lacking the enzyme was taken.

3.2. Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using 15 % separating and 5 % stacking gels. Protein bands were visualized by overnight staining with 0.1 % (w/v) Coomassie Brilliant Blue R 250. The gels were destained using 40 % (v/v) methanol/ 10 % (v/v) acetic acid. Commercial SOD gave a single band in the SDS-PAGE and was hence used without further purification in the experiments.

3.3. Immunization of rabbits and purification of IgG

Antibodies were raised in the rabbits by injecting SOD emulsified in Freund's complete/incomplete adjuvant. Healthy male albino rabbits, weighing 2.5-3 kg received subcutaneously 300 µg of antigen dissolved in 0.5 ml of 20 mM sodium phosphate buffer, pH 7.2, mixed and emulsified with equal volume of Freund's complete adjuvant (FCA) as first dose. The animals were rested for 21 days and then boosted weekly with 150 µg of antigen in 0.5 ml of sodium phosphate buffer,

emulsified with equal volumes of Freund's incomplete adjuvant (FIA), for three consecutive weeks. Five days after final booster, the animals were bled through a marginal ear vein and blood (30 ml) was allowed to clot at room temperature for 6-8 h. Serum was then collected by centrifugation at 1600 x g for 15 min, decomplemented at 56°C for 30 min and preserved at -20°C. Formation of SOD specific antibodies was monitored by ELISA. IgG from the serum of immunized rabbits was purified to homogeneity on DEAE-cellulose matrix after ammonium sulphate precipitation according to the procedure followed in our laboratory (Rehan and Younus, 2006).

3.4. Enzyme-linked immunosorbent assay (ELISA)

The cross-reactivity of the antibodies with SOD was determined by ELISA. Ninety six-well microtitre plates (Roll Piove di Sacco, Italy) were coated overnight with 100 µl of SOD (5 µg/ml) in 0.05 M carbonate-bicarbonate buffer, pH 9.6 at 4°C. After extensive washing with phosphate buffered saline (PBS)-Tween 20 buffer, 150 µl of blocking buffer (5% BSA in PBS) was applied to the wells and the plates incubated at 37°C for 2 h. After removal of the blocking buffer, 100 µl of anti-SOD IgG (16 µg/ml) was added and the binding was allowed to proceed at 37°C for 2 h. The microtitre plates were washed and incubated with 100 µl of HRP conjugated goat anti-rabbit IgG at 37°C for 1 h. After the usual washing steps, the peroxidise reaction was initiated by the addition of the substrate OPD/H_2O_2, arrested by the addition of 1 M H_2SO_4, and absorbance at 490 nm measured in an ELISA reader.

3.5. *In vitro* glycation of SOD by glucose, MG or a combination of both

SOD was dissolved in 20 mM sodium phosphate buffer, pH 7.4 to make a stock of 1 mg/ml. Stock was then stored at -20°C for future use. In order to induce glycation, SOD (0.2 mg/ml) was incubated along with 0.5 M glucose, 10 mM MG or a combination of 0.5 M glucose and 10 mM MG in 20 mM sodium phosphate buffer pH 7.4 containing 0.15 M NaCl. Incubations were performed in autoclaved tubes in order to maintain sterile conditions during the prolonged incubations. No bacterial growth was detected during the periods of incubation. The incubations were carried out in a shaking water bath at 37°C for 1 h, 1 day, 5 days and 10 days. The existence of free glucose or MG in the SOD protein solutions after incubation was found to have no effect on the enzyme activity at room temperature. Therefore, these solutions

were not dialyzed after incubation. The native SOD incubated with phosphate buffer (20 mM, pH 7.4 containing 0.15 M NaCl) alone served as the control. Glucose or MG or combination of both glucose and MG-induced glycation of SOD was assessed by activity, SDS-PAGE, ELISA, absorption, intrinsic fluorescence, AGEs specific fluorescence, thioflavin (ThT) fluorescence and Far-UV circular dichroism (CD) spectroscopic studies.

3.6. Preparation of stock solutions of natural products

A. vera plants were harvested in the month of November from the plant nursery, Aligarh Muslim University, Aligarh, India. The fresh leaves were dissected into two halves and the inner colourless, mucilaginous pulp was homogenized in an electrical blender, centrifuged at 10,000 g at 4°C for 15 min to remove the fibres. The resultant supernatant was filtered, freeze dried at -20°C and lyophilized. To make a stock solution of *A. vera* extract, 30 mg of the lyophilized powder was dissolved in 100% DMSO. 5 mM stocks of TQ, aloin and EA, each were freshly prepared in 100% DMSO. 5 mM stock alliin was prepared in 20 mM phosphate buffer, pH 7.4.

3.7. Effect of natural products on the glycation of SOD

SOD (0.2 mg/ml) in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl was incubated with 0.5 M glucose or 10 mM MG or combination of 0.5 M glucose and 10 mM MG for 10 days at 37°C in the presence of 0, 10, 20 and 50 µg/ml of *A. vera* extract or 0, 10, 20 and 50 µM TQ, aloin, EA or alliin. Since, TQ, aloin and EA were dissolved in DMSO, the final DMSO concentration in the incubation mixture was 1%. The effect of *A. vera* extract, TQ, aloin, EA and alliin on the glycation of SOD with glucose or MG or a combination of both were assessed by activity, SDS-PAGE, ELISA, absorption, intrinsic fluorescence, AGEs specific fluorescence and ThT fluorescence spectroscopic studies. Far-UV CD studies were performed for Alliin but not for other natural products as DMSO in these samples interfered with the measurements in this wavelength range.

3.8. Biophysical studies on the glycation of SOD and its protection by natural products

3.8.1. Absorption Spectroscopy

Absorbance measurements were carried out on a double beam Perkin Elmer spectrophotometer (Lambda 25). The absorbance measurements in the case of glycation of SOD and its protection by TQ were performed using an old UV lamp that got changed in the latter experiments, therefore, the values of absorbance were much lower in the experiments on the glycation of SOD and its protection by TQ as compared to the latter experiments. The spectra of SOD (0.2 mg/ml) in absence/presence of glucose or MG and absence/presence of TQ, *A. vera*, aloin, EA or alliin were measured in the wavelength range of 240-500 nm.

3.8.2. Fluorescence Spectroscopy

All fluorescence measurements were carried out on a Shimadzu spectrofluorometer (model RF-5301PC). The fluorescence measurements in the case of glycation of SOD and its protection by TQ were performed using an old fluorescent lamp that got changed in the latter experiments, therefore, the values of fluorescence intensity were much lower in the experiments on the glycation of SOD and its protection by TQ as compared to the latter experiments. The intrinsic fluorescence of SOD (0.2 mg/ml) incubated alone, with glucose or with MG in the absence/presence of TQ, *A. vera* extract, aloin, EA or alliin was monitored with excitation at 280 nm and emission in the range 290-400 nm. The slit widths were 5 nm for both excitation and emission. The formation of fluorescent AGE products was monitored with excitation at 350 nm and emission in the range 400-480 nm. The slit widths were 3 nm for both excitation and emission. The fibrillar state of incubated SOD was determined via ThT, a reagent used for detecting the β-sheet configuration in proteins (Schmitt *et al.*, 2005). The fluorescence of the above incubation mixtures was monitored after adding 6 μM ThT reagent at excitation wavelength of 440 nm and the emission was measured in the range 450-600 nm. The slit widths were 10 nm for both excitation and emission.

3.8.3. CD Spectroscopy

Far-UV CD measurements were carried out with a Jasco spectropolarimeter (J-815) equipped with a Jasco Peltier-type temperature controller (PTC-424S/15). The

instrument was calibrated with D-10-camphorsulphonic acid. The spectra were collected in a cell of 0.1 cm with scan speed of 100 nm/min and response time of 1 S. Each spectrum was the average of 2 scans.

No. of replicates ??
Standard deviation ??
Statistical analysis ??

Results and Discussion

4. Results and Discussion

4.1. SOD purity and activity

SOD is a dimeric protein of molecular mass of 32 kDa (Bannister *et al.*, 1971). The enzyme migrated as a single band (16 kDa) (Fig. 14) in SDS-PAGE and hence, it was homogenous and, therefore, it was used in the experiments without further purification.

The activity of SOD was determined by PMS-NADH-NBT assay under standard assay conditions. The observed spontaneous NBT reduction by $O_2^{\cdot-}$ was inhibited in a concentration-dependent manner up to 3 µg by SOD (Fig. 15). The enzyme activity increased linearly up to 0.5 µg of the enzyme, after which the increase in activity was slow.

4.2. *In vitro* glycation of SOD by glucose, MG or both

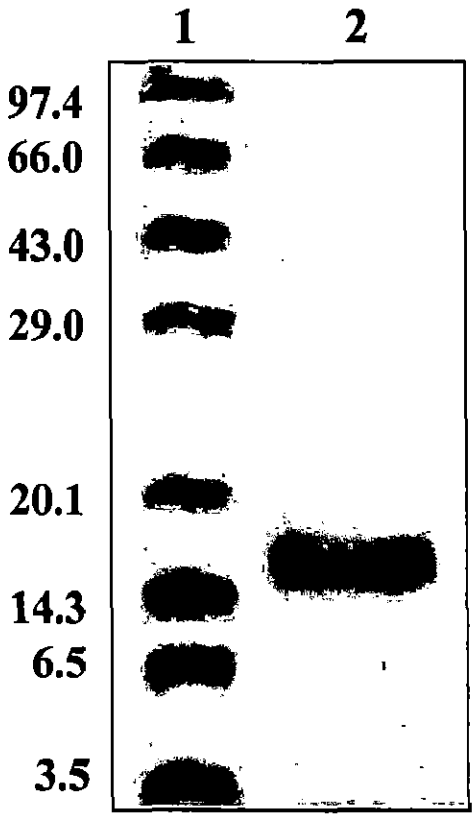
It has been reported that prolonged incubation of enzymes with reducing sugars results in glycation and hence inactivation (Monnier and Cerami, 1981; Brownlee *et al.*, 1984; Wells-Knetch *et al.*, 1995). MG is a highly reactive α-oxoaldehyde that plays an important role in glycation reactions, formation of AGEs and other complications associated with hyperglycemia and related disorders (Jabeen *et al.*, 2006). SOD is possibly the most important antioxidant enzyme that is specifically involved in the detoxification of $O_2^{\cdot-}$, enabling cells to cope with lethal oxidative environments. SOD itself has been shown to undergo glycation by reducing sugars and MG and hence inactivation (Jabeen *et al.*, 2006; Jabeen and Saleemuddin, 2006). SOD was incubated with high non-physiological concentration of glucose (0.5 M) or MG (10 mM) enzyme which may serve as an appropriate model for the long-term effects of glucose or MG on the enzyme (Jabeen *et al.*, 2006; Coussons *et al.*, 1997). Glycation-induced changes in the enzyme were evaluated by activity, SDS-PAGE, ELISA, UV absorption, fluorescence and CD studies.

4.2.1. Activity studies

The effect of 0.5 M glucose or 10 mM MG or a combination of 0.5 M glucose and 10 mM MG on the activity of SOD is shown in Fig. 16. Incubation with glucose at 37°C resulted in a decrease in the activity of SOD. At the end of ten days of

Fig. 14. SDS-PAGE of SOD. Commercial SOD was analyzed by 15% SDS-PAGE. Molecular weight markers (Genei) were applied to lane 1. Lane 2 contained 10 μg of SOD. The gels were stained with coomassie brilliant blue.

Fig. 15. Inhibition of NBT reduction by SOD under aerobic conditions. Different amounts of purified SOD were added to the reaction mixture containing NBT and NADH under standard assay conditions. The reaction was initiated with the addition freshly prepared PMS and was monitored for 120 seconds at 560 nm.


1
2
97.4
66.0
43.0
29.0
20.1
14.3
6.5
3.5

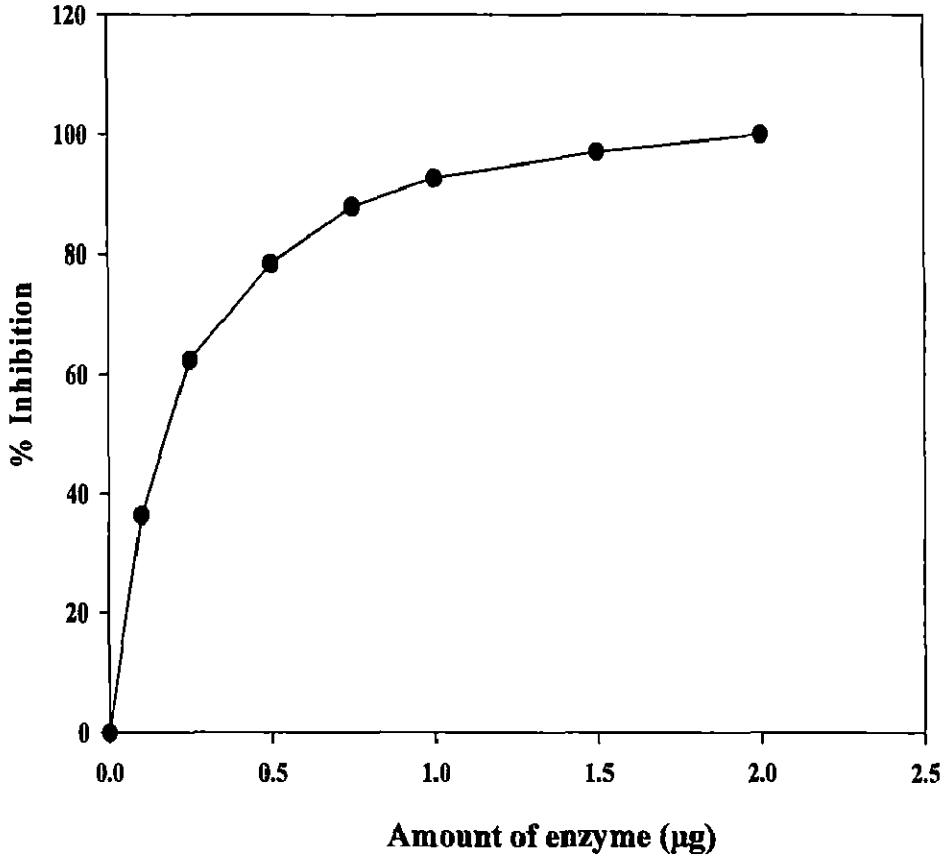
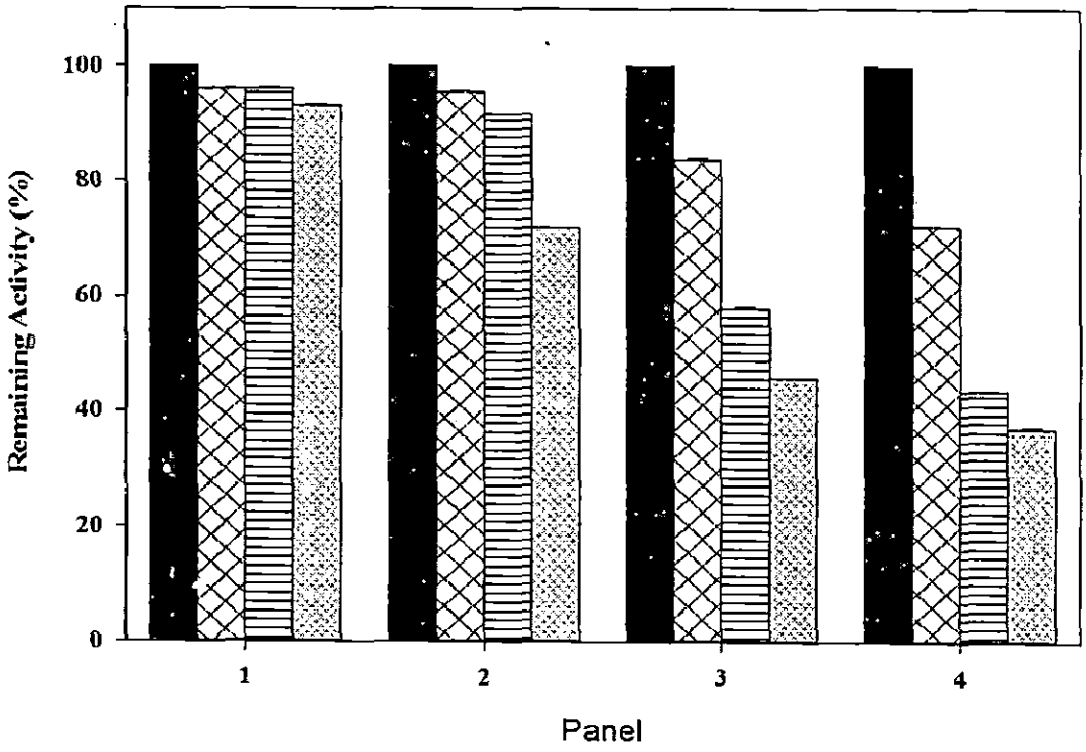

120
100
80
60
40
20
0
% Inhibition
0.0
0.5
1.0
1.5
2.0
2.5
Amount of enzyme (µg)

Fig. 16. Effect of glycation on the activity of SOD. SOD (0.2 mg/ml) was incubated alone (Panel 1), with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or combination of 0.5 M glucose and 10 mM MG (Panel 4) for 1 (♯), 5 (≡) and 10 (▒) days at 37°C. Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■). The enzyme activity was then determined under standard assay conditions.



No. of replicates ?

statistical analysis ?

? cross-linked or degraded products must be visible in the SDS-PAGE. This is not clear from fig. 17A

incubation with glucose, the resultant activity was 72% of the control. MG, which is more reactive than glucose in the Maillard reaction inactivated SOD more rapidly. At the end of ten days of incubation with MG or a combination of glucose and MG, the residual activity was 46% and 37% of the control, respectively. SOD did not lose significant activity when incubated alone i.e., in the absence of glucose or MG. The residual activity in this case was 93% after 10 days of incubation.

4.2.2. SDS-PAGE

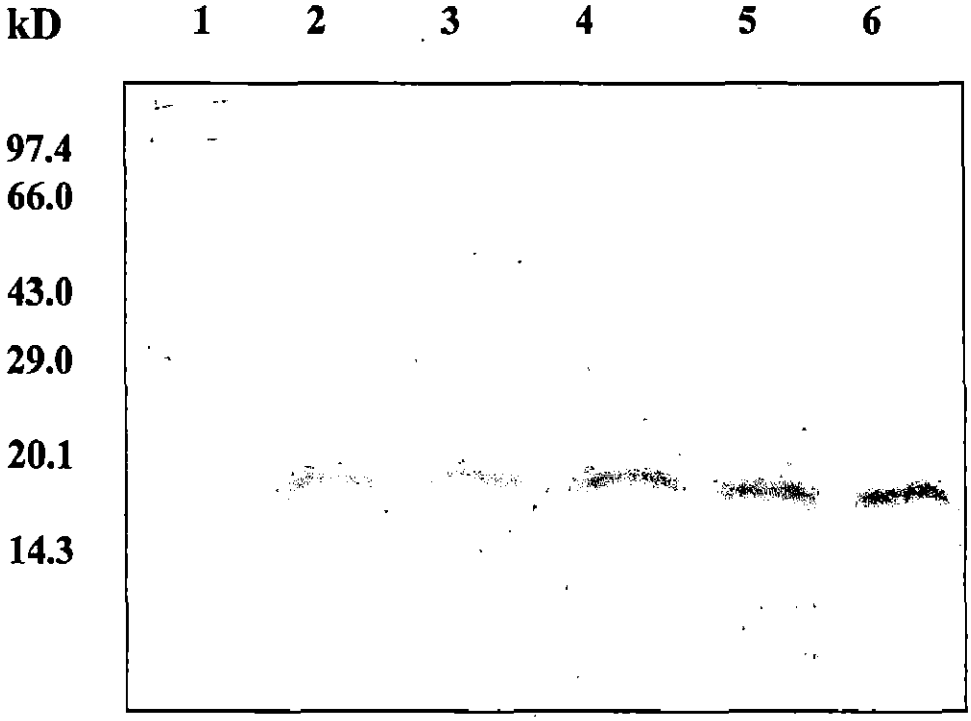
SDS-PAGE of SOD incubated at 37°C for various days in the absence of glucose or MG exhibited very slight decrease in the intensity of enzyme band (Fig. 17A). This is correlated with the activity results that have shown the retention of 93% activity after 10 days of incubation for SOD incubated alone. However, SOD incubated with glucose revealed a significant decrease in in the staining intensity of the band corresponding to the enzyme (Fig. 17B). The band appeared more lighter with an increase in the duration of exposure to the sugar, presumably indicating its crosslinking and/or degradation into small peptides. Formation of high molecular weight cross-linked aggregates in the SOD exposed to MG or a combination of MG and glucose is evident from figure 17C and 17D, respectively. The high molecular weight bands started appearing with progressive increase of incubation time and their intensity increased with days of incubation. After ten days of incubation, no band corresponding to the free enzyme (uncross linked) was visible in the gel. The effect of MG is thought to be due to its ability to form stable heterocyclic compounds that cross-link protein. These changes in the enzyme preparation exposed to both glucose and MG were slightly faster as compared to that exposed only to MG. However, the effect of MG dominates over the effect of glucose, since MG is much more reactive and potent glycating agent as compared to glucose.

4.2.3. ELISA

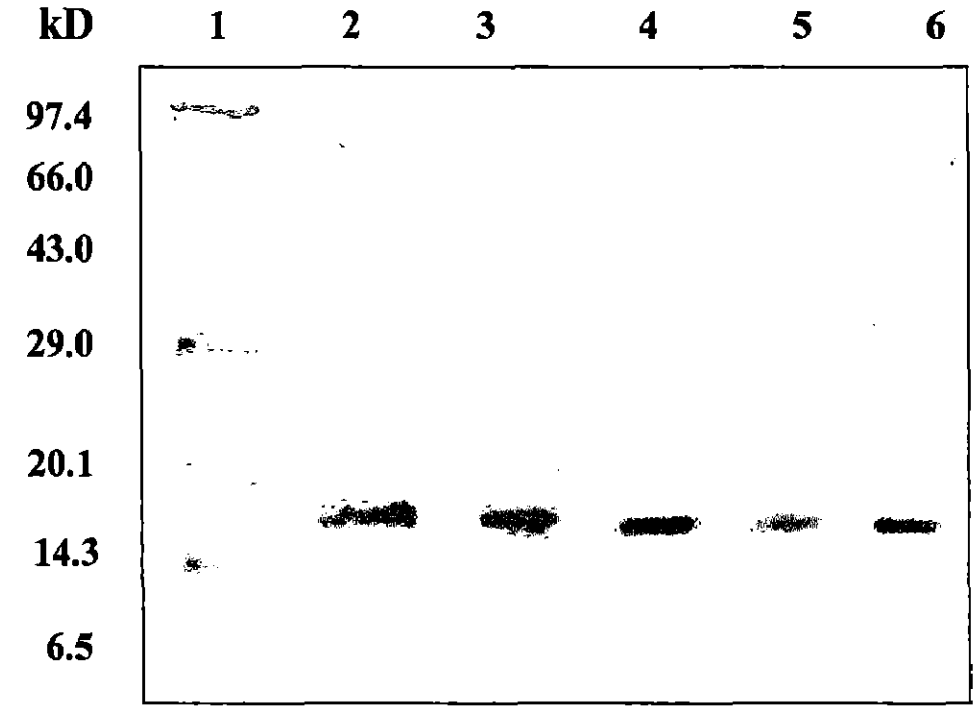
Incubation of SOD alone or with glucose, MG and both glucose and MG results in a decrease in absorbance at 490 nm in ELISA, indicating reduced cross-reactivity with anti-SOD antibodies, which we believe is due to the structural/ chemical modification of the epitopes of enzyme due to incubation at 37°C and by glycation (Fig. 18). When SOD is incubated for increasing days at 37°C with glucose (Fig. 18, Panel 2), MG (Fig. 18, Panel 3) or both glucose and MG (Fig. 18, Panel 4),

Fig. 17. SDS-PAGE of SOD incubated alone (A), with 0.5 M glucose (B), with 10 mM MG (C) or a combination of 0.5 M glucose and 10 mM MG (D) for various time periods at 37°C. Lane 1 shows molecular weight markers (Genei); Lanes 2, 3, 4, 5 and 6 show SOD (10 μg) incubated alone, with glucose, with MG or with a combination of glucose and MG for 0 h, 1h, 1 day, 5 days and 10 days, respectively.

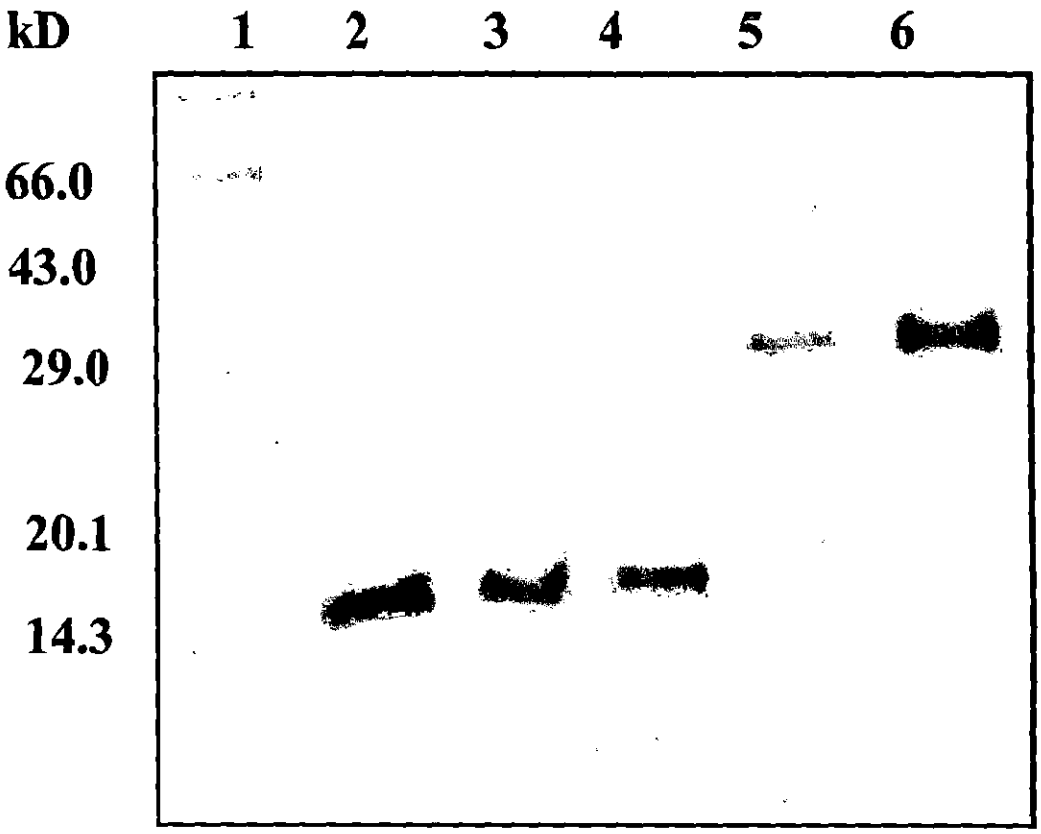
A


kD
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3

B


kD
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3
6.5

C



D

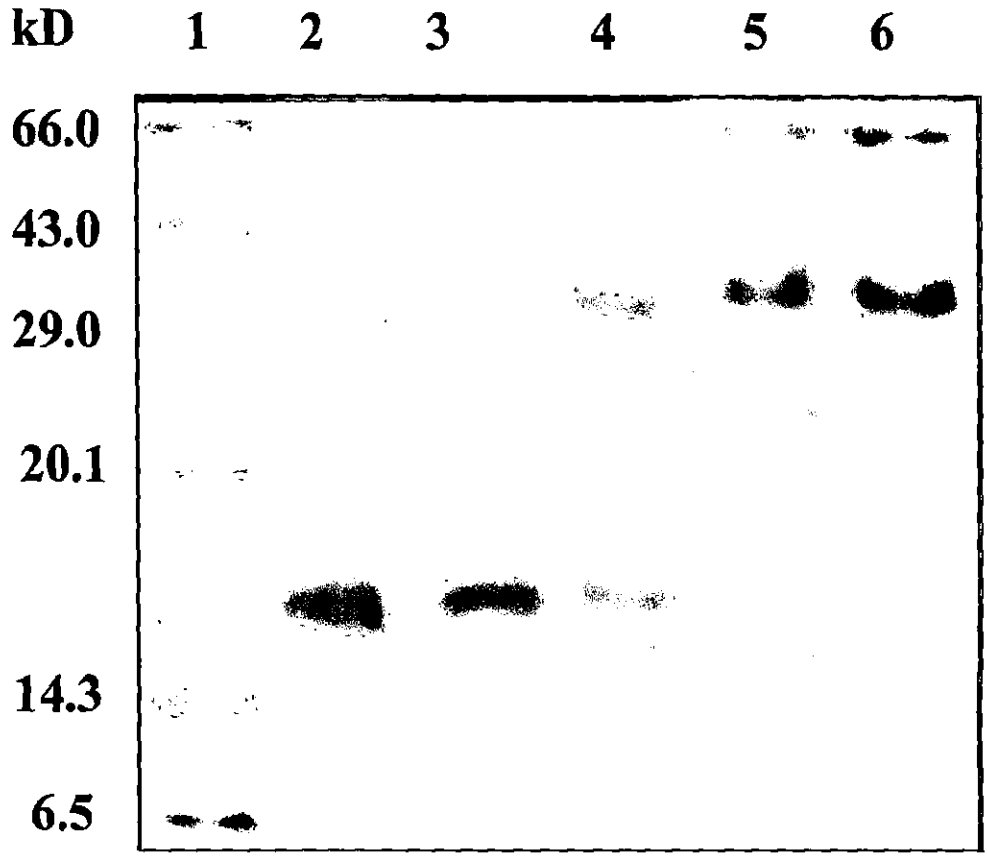
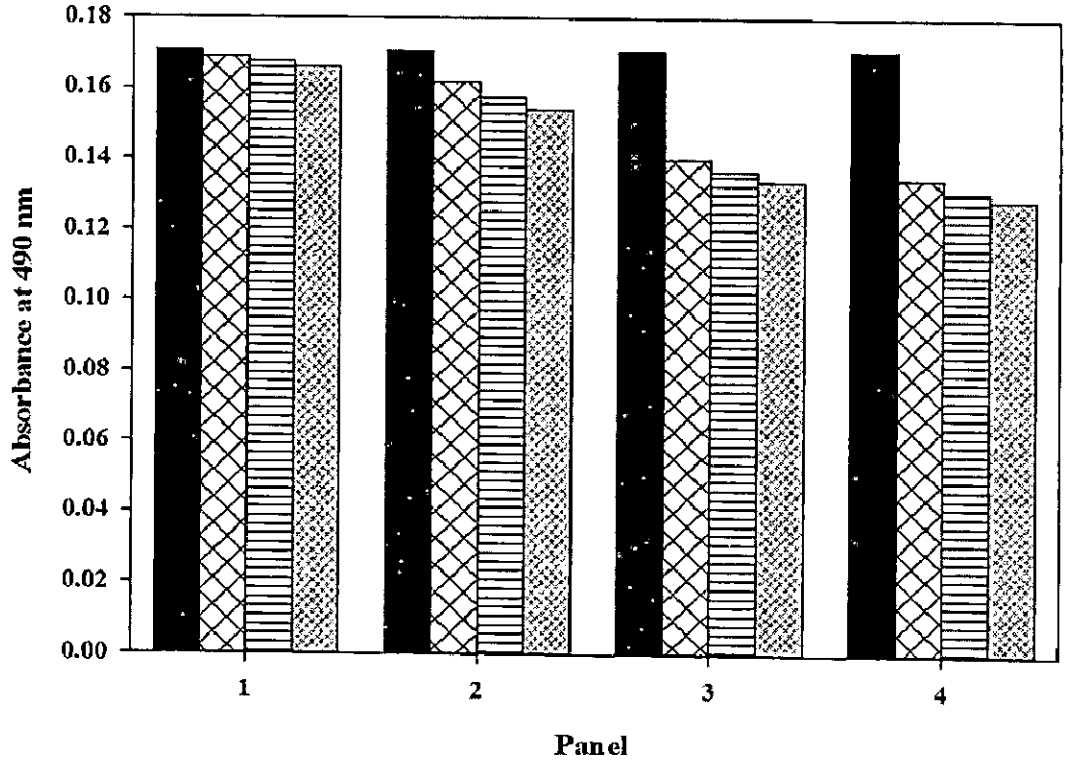


Fig. 18. Effect of glycation of SOD on the cross-reactivity (ELISA) of anti-SOD antibodies with the enzyme. SOD (0.2 mg/ml) was incubated alone (Panel 1), with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or combination of 0.5 M glucose and 10 mM MG (Panel 4) for 1 day (♯), 5 days (≡) and 10 days (▒) in 20 mM sodium phosphate buffer, pH 7.4 at 37°C. Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■). Each value represents the average for two independent experiments performed in triplicates.

should be
at least 3 replicates?


0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance at 490 nm
1
2
3
4
Panel

for 1 day, 5 days and 10 days, a progressive decrease in absorbance at 490 nm (cross-reactivity with anti-SOD antibodies). Therefore, this experiment shows that glycation induces structural/chemical changes in enzyme incubated with glucose, MG or a combination of both, at 37°C. Native SOD incubated till 10 days at 37°C (control), no or very slight decrease in absorbance was observed (Fig. 18, Panel 1).

4.2.4. UV absorption studies

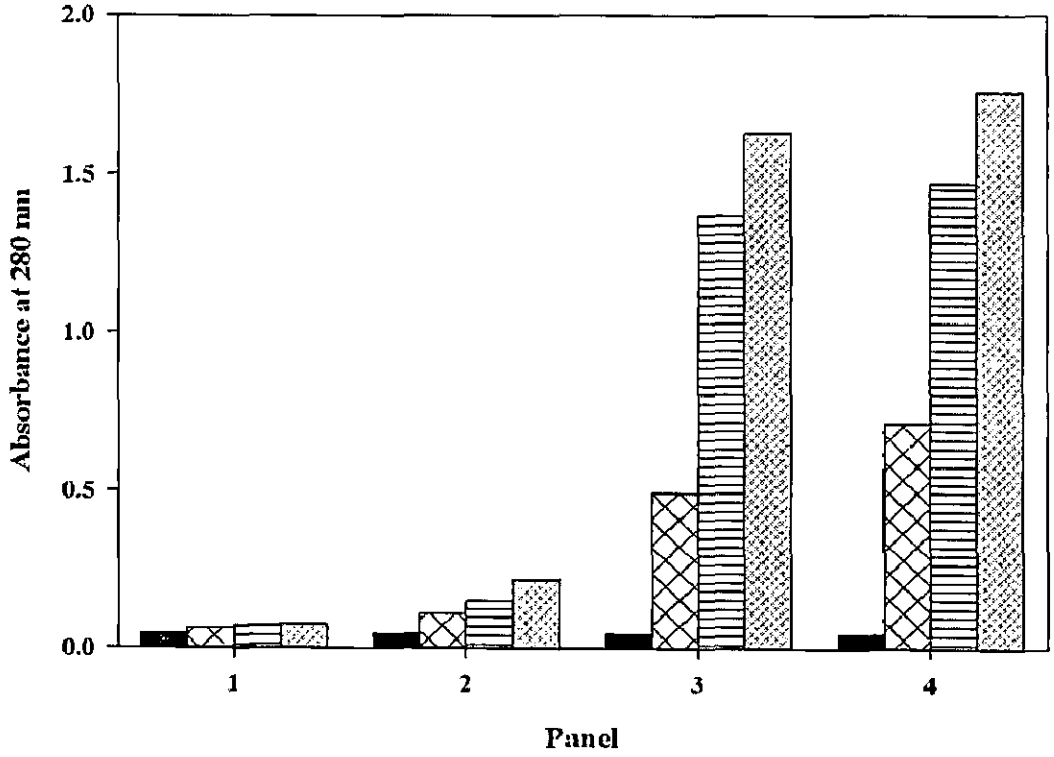
Bovine erythrocyte Cu,Zn-SOD lacks tryptophan residues and has one tyrosine residue per subunit. SOD that had not been incubated with glucose or MG (native SOD) gave absorbance in the wavelength range 250-300 nm, whereas in glucose, MG or a combination of glucose and MG glycated (Fig. 19) samples with increasing days of incubation, an increasing hyperchromicity was observed. Native SOD incubated till 10 days at 37°C (control), no or very slight increase in absorbance at 280 nm was observed (Fig. 19, Panel 1). The increase in absorbance was much more and faster in the case of SOD incubated with MG than with glucose, implying that the structural changes in the enzyme were much more and faster with MG than with glucose (Fig. 19, Panel 3 and 2, respectively). And the increase in absorbance was the most and fastest in the case of SOD incubated with a combination of both glucose and MG (Fig. 19, Panel 4) and is similar to the effect of MG alone since MG is a stronger glycating agent than glucose hence the effect of MG dominates over the effect of glucose. The observed hyperchromicity in the case of glucose incubated SOD could be due to modification of aromatic amino acids or changes in the micro environment of aromatic amino acids of the enzyme. The observed hyperchromicity in the case of MG or glucose + MG could be due to change in the conformation of SOD due to glycation induced unfolding leading to cross-liking and aggregation.

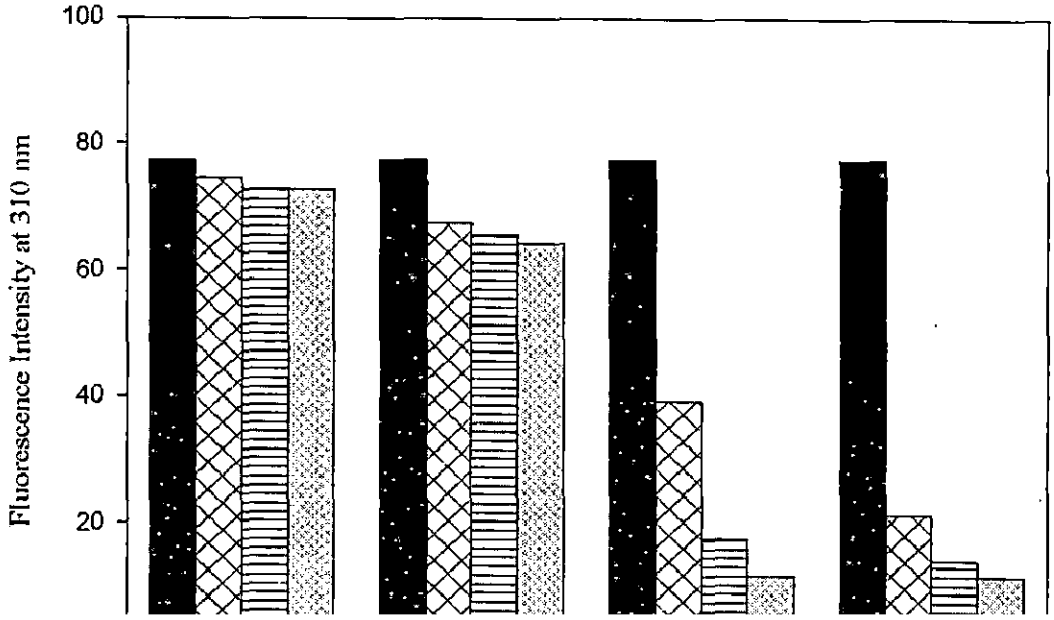
4.2.5. Intrinsic fluorescence studies

Total intrinsic fluorescence of native and glycated SOD was measured by exciting at 280 nm. Native SOD gave an emission peak at 310 nm, whereas in glucose, MG or a combination of glucose and MG glycated samples with increasing days of incubation, an increasing fluorescence quenching was observed (Fig. 20). In the control sample, no or very slight fluorescence quenching at 310 nm was observed (Fig. 20, Panel 1). Therefore, glycation induced structural changes in SOD incubated with glucose or MG or a combination of both (fFg. 20, Panel 2, 3 and 4, respectively).

Fig. 19. Effect of glycation of SOD on the absorbance of the enzyme. Absorbance at 280 nm for samples of SOD (0.2 mg/ml) incubated for 1 day (♯), 5 days (≡) and 10 days (▒) in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or with a combination of 0.5 M glucose and 10 mM MG (Panel 4). Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■). Each value represents the average for two independent experiments performed in triplicates.

Fig. 20. Effect of glycation of SOD on the intrinsic fluorescence of the enzyme. Fluorescence intensity at the excitation/emission wavelengths of 280/310 nm for samples of SOD (0.2 mg/ml) incubated for 1 day (♯), 5 days (≡) and 10 days (▒) in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or with a combination of 0.5 M glucose and 10 mM MG (Panel 4). Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■). Each value represents the average for two independent experiments performed in triplicates.


2.0
1.5
1.0
0.5
0.0
Absorbance at 280 nm
1
2
3
4
Panel


100
80
60
40
20
Fluorescence Intensity at 310 nm

Fluorescence quenching was much more and faster for MG than for glucose incubated enzyme, implying again that MG incubation has a greater effect on the structure of SOD than glucose incubation. And incubation of SOD with both glucose and MG resulted in even more and faster fluorescence quenching than MG alone incubated enzyme implying greatest structural change in this case.

4.2.6. AGEs specific fluorescence studies

We have followed the formation of AGEs in the samples via autofluorescence (Schmitt *et al.*, 2005). Fluorescent AGEs specific fluorescence of native and glycated SOD was measured by exciting at 350 nm. The control sample showed no fluorescence in the wavelength range 400-480 nm. Whereas SOD incubated with glucose, MG or a combination of glucose and MG showed AGEs specific fluorescence in the wavelength range 400-480 nm which increased with increasing days of incubation (Fluorescence enhancement) (Fig. 21, Panel 2, 3, 4, respectively). The fluorescence enhancement observed in the case of glucose incubated SOD was low, implying few AGEs formed in this case (Fig. 21, Panel 2). However, the enhancement was much more and faster in the case of MG incubated enzyme, implying formation of larger quantity of AGEs (Fig. 21, Panel 3). And incubation of SOD with both glucose and MG resulted in even more and faster fluorescence enhancement than MG alone incubated enzyme implying greatest quantity of AGEs formed in this case (Fig. 21, Panel 4). The spectra of fluorescence intensity versus wavelength (400-480 nm) were rather broad (data not shown), and this probably reflects the presence of a number of different fluorescent compounds being formed during glycation.

4.2.7. ThT fluorescence studies

ThT is a dye that interacts with the fibrillar structure of proteins, upon interaction its fluorescence intensifies, while in its free form is only weakly fluorescent. This quality has been employed in the detection of amyloid fibril structures in proteins (Schmitt *et al.*, 2005). The control sample showed very slight enhancement in the fibrillar state (Fig. 22, Panel 1). Whereas SOD incubated with glucose, MG or a combination of glucose and MG showed enhancement in the fibrillar state, which increased with increasing days of incubation (Fluorescence enhancement) (Fig. 22, Panel 2, 3, 4, respectively). The fibrillar state enhancement

Fig. 21. Effect of glycation of SOD on the AGEs specific fluorescence of the enzyme. AGEs specific fluorescence intensity at the excitation/emission wavelengths of 350/450 nm for samples of SOD (0.2 mg/ml) incubated for 1 day (♯), 5 days (≡) and 10 days (▒) in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or with a combination of 0.5 M glucose and 10 mM MG (Panel 4). Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■).

Fig. 22. Effect of glycation of SOD on the ThT fluorescence of the enzyme. ThT fluorescence intensity at the excitation/emission wavelengths of 440/480 nm for samples of SOD (0.2 mg/ml) incubated for 1 day (♯), 5 days (≡) and 10 days (▒) in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or with a combination of 0.5 M glucose and 10 mM MG (Panel 4). Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■).

Fluorescence Intensity at 480nm
0
30
60
90
120
150
180
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Panel

Fluorescence Intensity at 450 nm
0
2
4
6
8
10
1
2
3
4
Panel

observed in the case of both glucose and MG incubated SOD was the most, followed by MG incubated enzyme, and then by glucose incubated enzyme. Therefore, fibrils were formed in all the three cases.

4.2.8. CD studies

Far-UV CD studies in the 200-250 nm wavelength range were performed to measure the changes in the secondary structure of SOD upon glycation. Native SOD gave a negative peak at 208 nm, whereas in glucose, MG or a combination of glucose and MG glycated samples with increasing days of incubation, a progressive decrease in the negative ellipticity was observed (Fig. 23, Panel 2, 3, 4, respectively). In the control sample, very slight decrease in negative ellipticity occurred (Fig. 23, Panel 1). Therefore, glycation induced changes in the secondary structure of SOD in all the above three cases. However, the decrease in negative ellipticity was more and faster for MG than for glucose incubated enzyme, implying that MG incubation has a greater effect on the secondary structure of SOD than glucose incubation. And incubation of SOD with both glucose and MG resulted in even more and faster secondary structural changes than MG alone.

4.3. Protective effect of TQ on the glycation of SOD with glucose or MG

4.3.1. Activity studies

The effect of TQ on the glycation of SOD by 0.5 M glucose or 10 mM MG was studied. Fig. 24 shows the increase in activity of SOD incubated for ten days at 37°C alone in the absence of glucose or MG and that incubated in the presence of glucose, MG or a combination of both and with increasing concentration of TQ. SOD incubated alone with TQ showed a slight increase in its activity *i.e.* the activity increased by 2% when the enzyme was incubated with 50 μM TQ as compared to the control (the sample that had no TQ) (Fig. 24, Panel 1). This 2% increase in activity of SOD is believed to be due the antioxidant property of TQ. SOD incubated with glucose, MG or a combination of both and TQ showed a greater increase in activity as compared to the enzyme that was not incubated with glucose or MG. The activity increased by 9.6%, 7.7% and 6.2% as compared to the control when the enzyme was incubated with glucose, MG or a combination of both glucose and MG, respectively, and TQ (Fig. 24, Panel 2, 3, 4, respectively). This observed further increase in activity

Fig. 23. Effect of glycation of SOD on far-UV CD of the enzyme. CD at 208 nm for samples of SOD (0.2 mg/ml) incubated for 1 day (♯), 5 days (≡) and 10 days (▒) in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or with a combination of 0.5 M glucose and 10 mM MG (Panel 4). Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■).

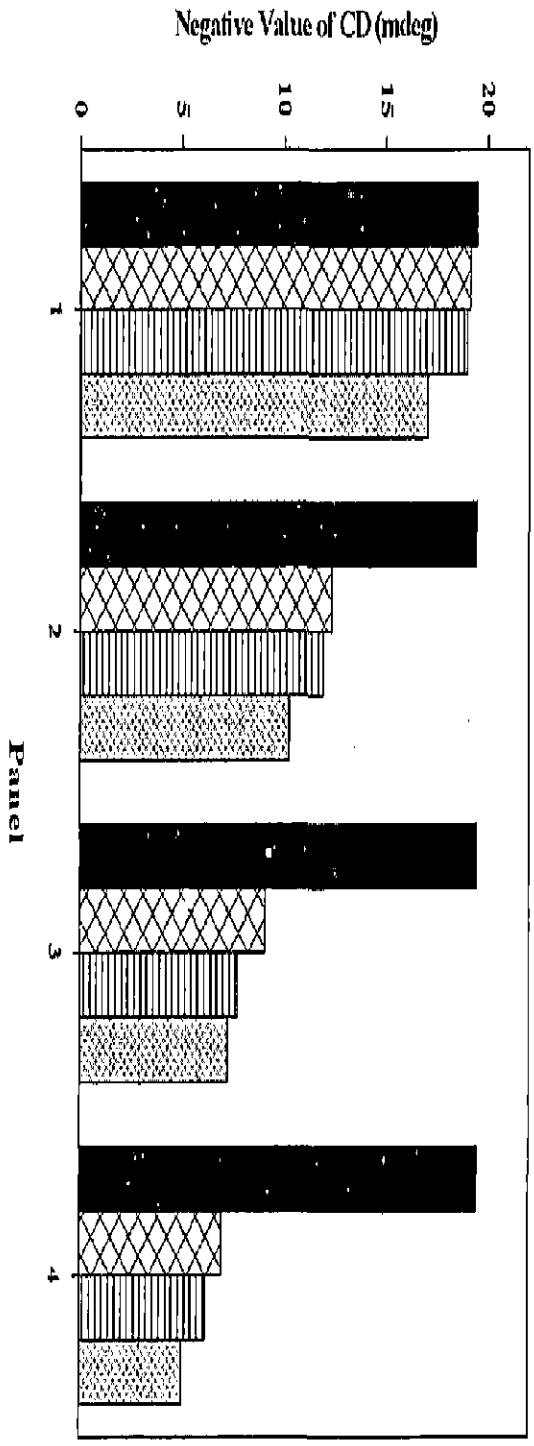

Negative Value of CD (mdeg)
0
5
10
15
20
Panel
1
2
3
4

Fig. 24. Effect of TQ on the activity of SOD incubated with glucose, MG or combination of both glucose and MG. SOD (0.2 mg/ml) was incubated alone (Panel 1), with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (#), 10 (≡), 20 (□) and 50 μM (▒) concentration of TQ. Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■). The enzyme activity was then determined under standard assay conditions. Each value represents the average for three independent experiments performed in duplicates.

Remaining Activity (%)
0
20
40
60
80
100
1
2
3
4
Phenol

is believed to be due to the antiglycating activity of TQ. The increase in activity was more in the case of glucose than for MG or MG and glucose. Therefore, it appears that TQ is a more effective antiglycating agent for sugars/compounds that are milder glycating agents.

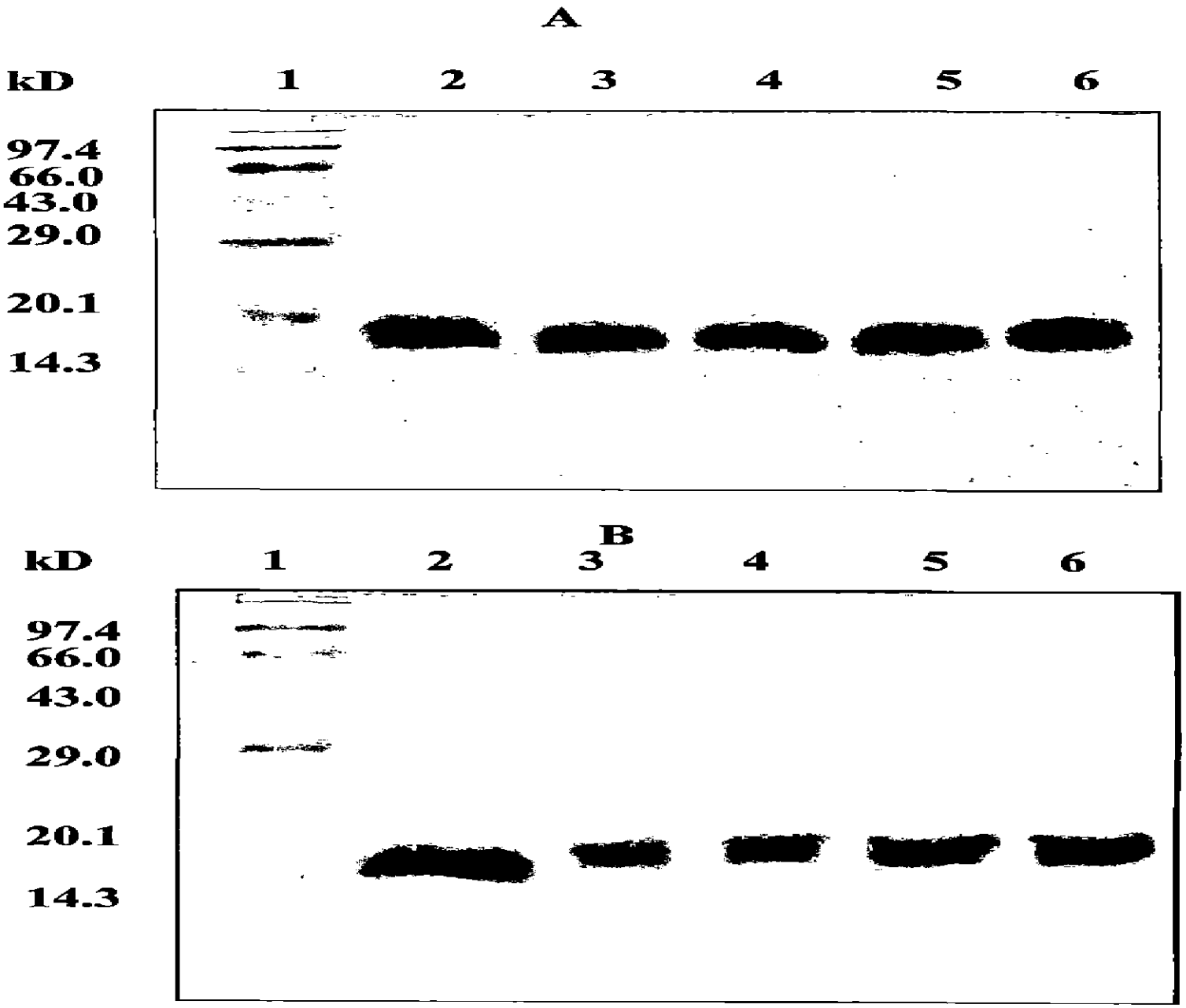
4.3.2. SDS-PAGE

The protective effect of TQ on SOD fragmentation/cross-linking induced by glycation is seen in Fig. 25. SDS-PAGE of SOD incubated for ten days in the absence of glucose or MG showed a slight increase in staining intensity with increasing TQ concentration (Fig. 25A). This correlates with 2% increase in activity with 50 μM TQ. However, the enzyme incubated for ten days with glucose showed a more increase in staining intensity with increasing TQ concentration (Fig. 25B). SOD incubated for ten days with MG (Fig. 25C) or a combination of both glucose and MG (Fig. 25D) exhibited a very slight decrease in the bands corresponding to the cross-linked aggregates with increasing TQ concentration. Therefore, it is evident from SDS-PAGE analysis that TQ slightly protected SOD against fragmentation/cross-linking induced by glycation. The protection appears to be in the following order for the enzyme incubated with glucose > MG > glucose + MG > no glycating compound. This correlates with the activity observations.

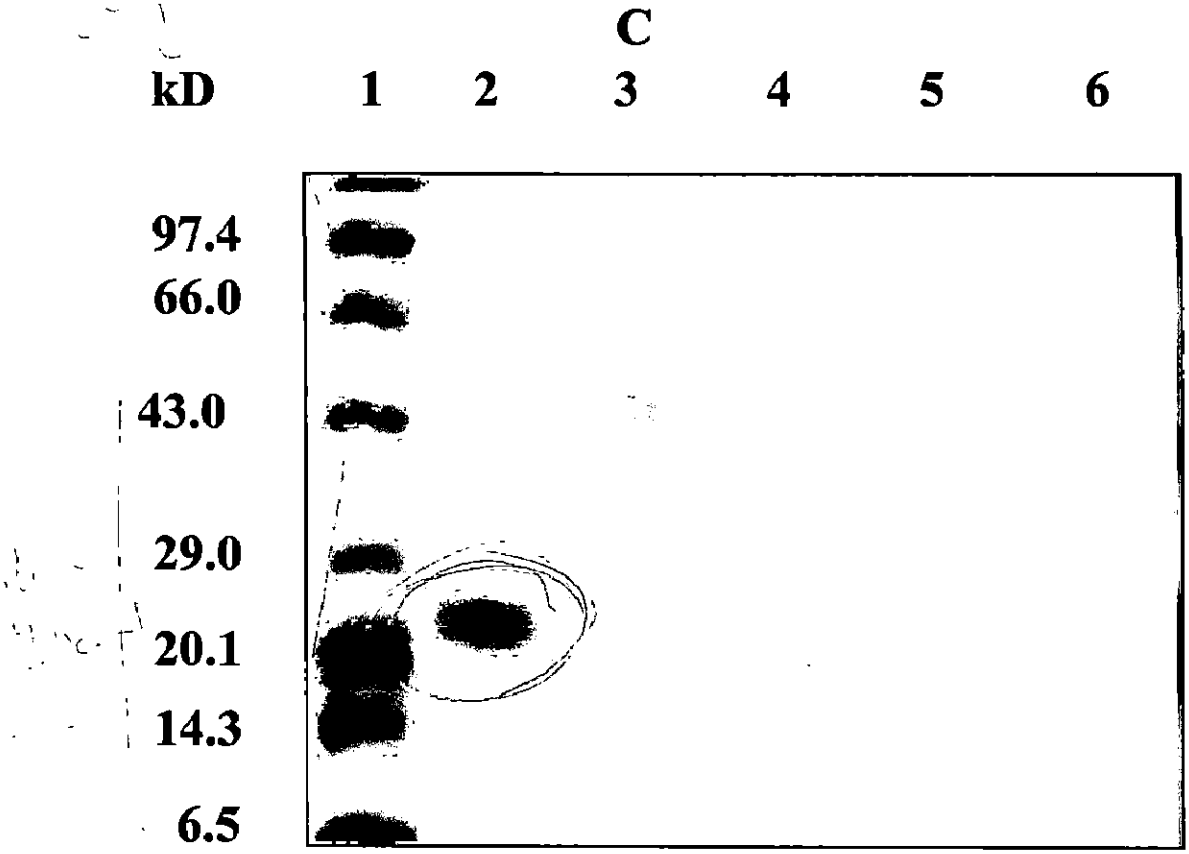
4.3.3. UV absorption studies

As observed earlier, glycation of SOD by glucose, MG and both glucose and MG results in hyperchromicity (structural changes) (Fig. 19). When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of TQ, a progressive decrease in absorbance at 280 nm with increasing TQ concentration was observed in all the three cases (Fig. 26, Panel 2, 3 and 4, respectively). SOD incubated alone for 10 days with increasing TQ concentration (control) exhibited no decrease in absorbance at 280 nm (Fig. 26, Panel 1). Therefore, TQ protected the enzyme to some extent against the structural changes induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of TQ. However, the enzyme was still far from the structure of the native enzyme even at 50 μM concentration of TQ in all the three cases. It was observed that the absorbance at 280 nm for samples of SOD incubated for 10 days with glucose, MG or both and without TQ but 1% DMSO was higher than

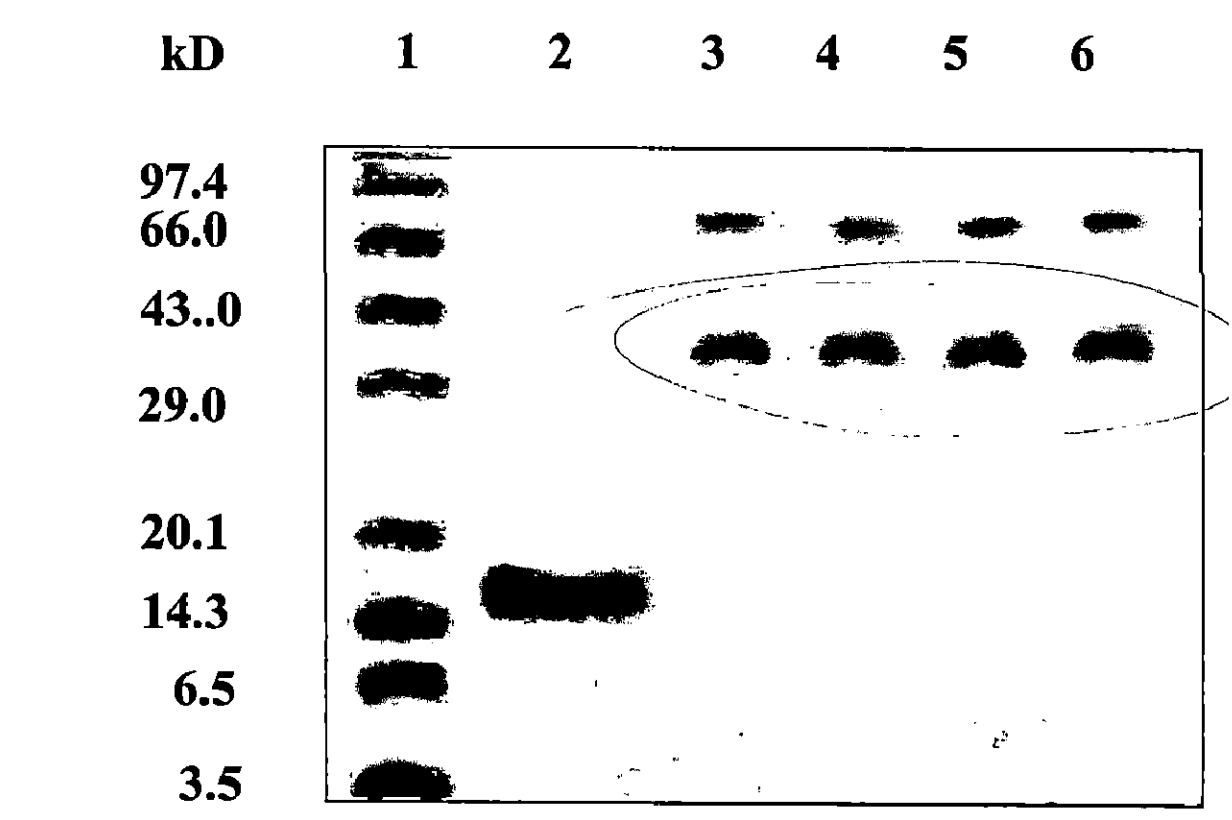
Fig. 25. SDS-PAGE of SOD incubated alone (A), with 0.5 M glucose (B), with 10 mM MG (C) or a combination of 0.5 M glucose and 10 mM MG (D) and with varying concentration of TQ for 10 days at 37°C. Lane 1 shows molecular weight markers (Genei); Lane 2 shows SOD (10 μg) that has not been incubated with glucose, MG or TQ. Lanes 3, 4, 5 and 6 show SOD (10 μg) incubated for 10 days alone or with glucose, MG or a combination of glucose and MG and with 0, 10, 20 and 50 μM TQ, respectively.


A
kD
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3
B
kD
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3

C


kD
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3
6.5

D


kD
1
2
3
4
5
6
97.4
66.0
43..0
29.0
20.1
14.3
6.5
3.5

the same samples incubated without TQ and DMSO. Therefore, it appears that even 1% DMSO present in the incubation mixtures decreases the stability of SOD at 37°C and hence makes it more prone to glycation. It has also been observed in the case of myoglobin and concanavalin A that low concentration of DMSO reduces the thermal stability of both proteins (Jackson and Mantsch, 1991).

4.3.4. Intrinsic fluorescence studies

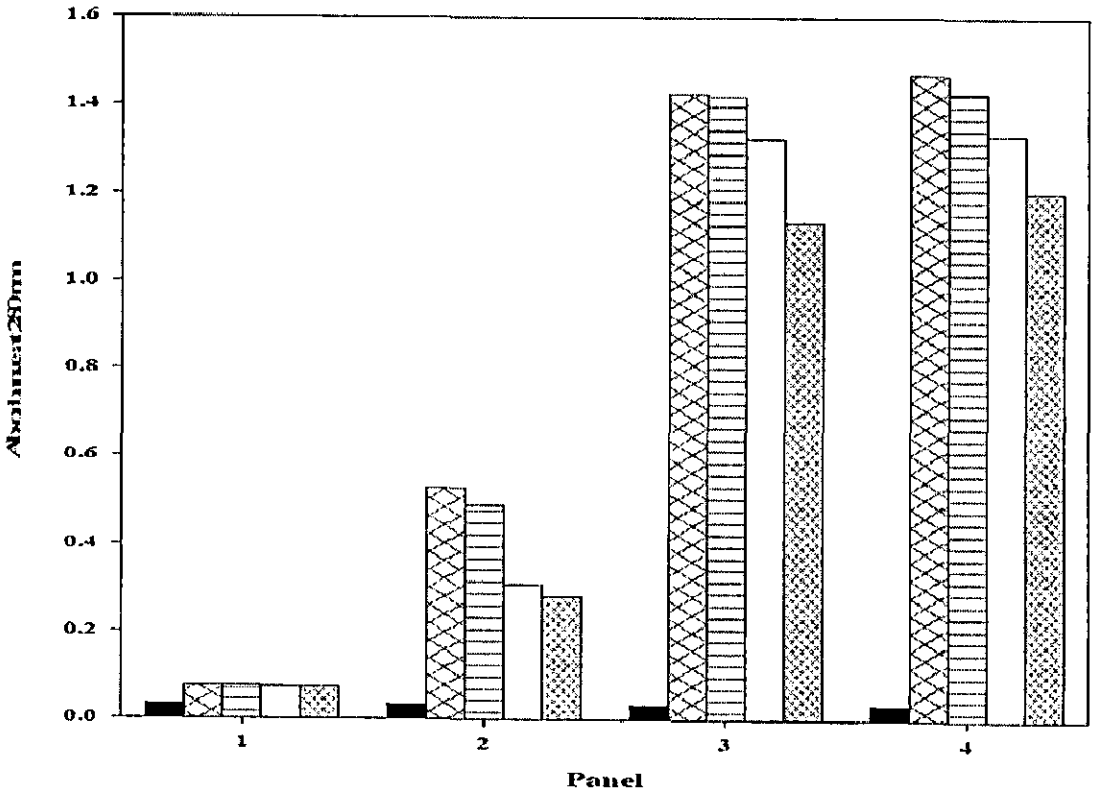
As observed earlier, glycation of SOD by glucose, MG and both glucose and MG results in intrinsic fluorescence quenching (structural changes) (Fig. 20). When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of TQ, a progressive increase in fluorescence at 310 nm with increasing TQ concentration was observed in all the three cases (Fig. 27, Panel 2, 3, 4). The control exhibited insignificant increase in fluorescence at 310 nm (Fig. 27, Panel 1). Therefore, again this experiment shows that TQ protected the enzyme to some extent against the structural changes induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of TQ. However, it was observed that SOD incubated for 10 days at 37°C with glucose, without TQ but with 1% DMSO showed fluorescence enhancement and not quenching. Therefore, it appears that DMSO somehow perturbs the environment around the aromatic residues of the protein which affects their fluorescence.

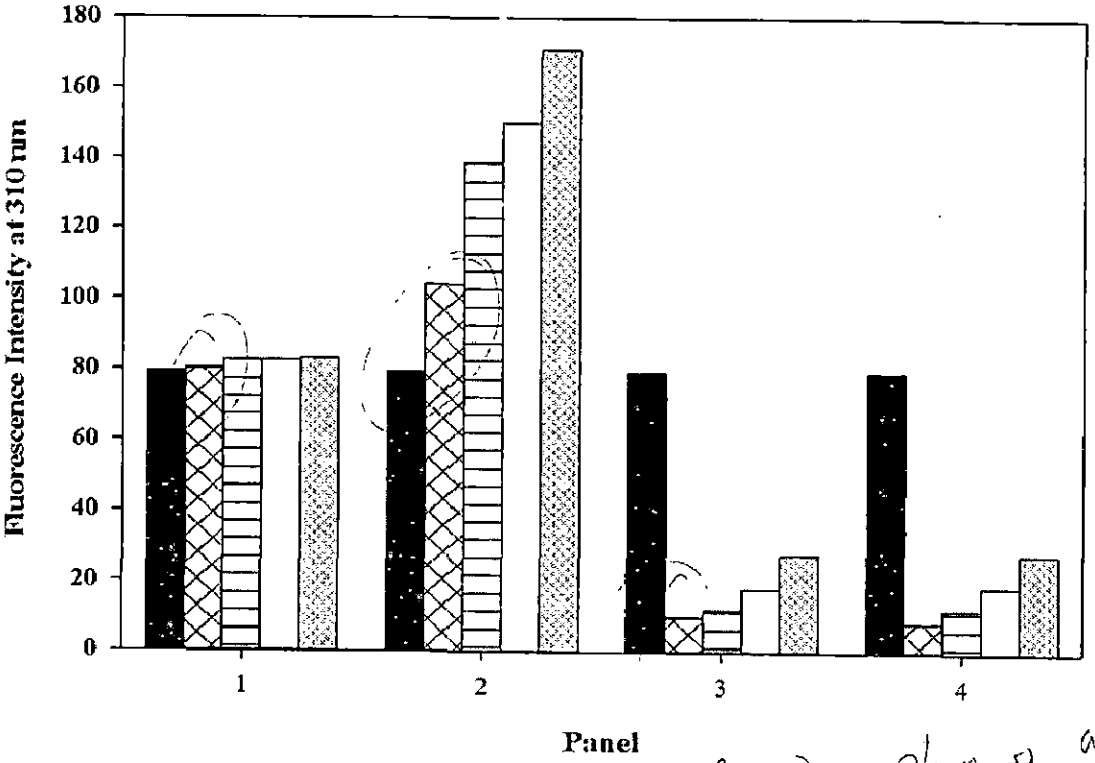
4.3.5. AGEs specific fluorescence studies

When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of TQ (Fig. 28, Panel 2, 3 and 4, respectively), a progressive decrease in AGEs specific fluorescence at 450 nm with increasing TQ concentration was observed in all the three cases. The control exhibited insignificant decrease in fluorescence at 450 nm (Fig. 28, Panel 1). Therefore, TQ protected the enzyme to some extent against formation of AGEs induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of TQ.

Fig. 26. Effect of TQ on the absorption changes induced in SOD due to glycation. Absorbance at 280 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM TQ. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).

Fig. 27. Effect of TQ on the intrinsic fluorescence changes induced in SOD due to glycation. Fluorescence intensity at the excitation/emission wavelengths of 280/310 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM TQ. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).





Lily Lane — in Panel 2 shows an ... in intrinsic fluorescence ... absence of TEA. Fig. 2b shows ... quenching ... the ...

4.3.6. ThT fluorescence studies

As observed earlier, glycation of SOD by glucose, MG and both glucose and MG results in ThT fluorescence enhancement (formation of fibrils) (Fig. 21). When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of TQ, a progressive decrease in ThT fluorescence at 480 nm with increasing TQ concentration was observed in all the three cases (Fig. 29, Panel 2, 3 and 4, respectively). The control exhibited insignificant decrease in ThT fluorescence at 480 nm (Fig. 29, Panel 1). Therefore, TQ protected the enzyme to some extent against formation of fibrils induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of TQ. It was again observed that the presence of 1% DMSO in the incubation mixtures decreases the stability of SOD at 37°C and hence makes it more prone to glycation and subsequent fibril formation.

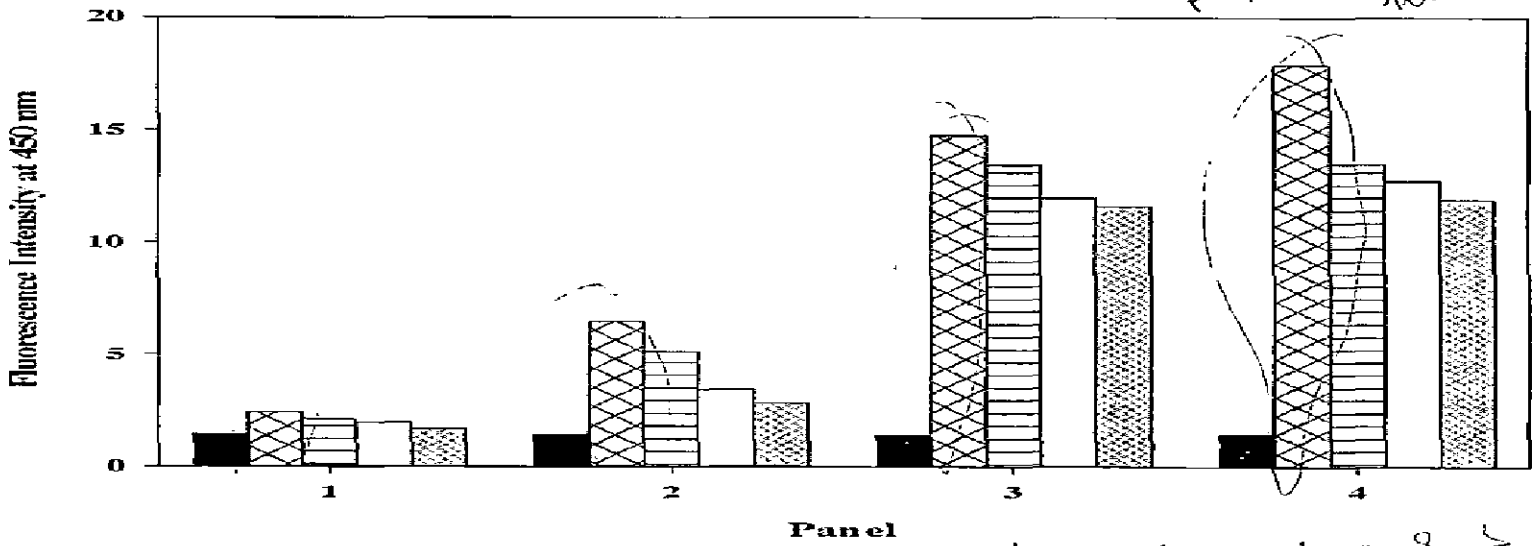
4.4. Protective effect of *A. vera* and aloin on the glycation of SOD with glucose or MG

4.4.1. Activity studies

The effect of *A. vera* extract and aloin on the activity of SOD glycated by 0.5 M glucose or 10 mM MG was studied. Fig. 30 shows the remaining activity of SOD incubated for ten days at 37°C alone, and that incubated in the presence of glucose, MG or a combination of both and with increasing concentration of *A. vera* extract (A) or aloin (B). SOD incubated for ten days alone with *A. vera* extract or aloin showed a slight increase in activity with increasing *A. vera* extract or aloin concentration (Fig. 30A and 30B, Panel 1). The activity increased by 4.4 and 5.4% when the enzyme was incubated with 50 µg/ml of *A. vera* extract or 50 µM aloin, respectively as compared to the control (the sample that had no *A. vera* extract or aloin). This increase in activity of SOD is believed to be due to the antioxidant property of *A. vera* extract and aloin. SOD incubated with glucose, MG or a combination of both, and *A. vera* extract or aloin showed a greater increase in activity as compared to the enzyme that was not incubated with glucose or MG. The activity increased by 10.0, 7.4 and 7.0% as compared to the control when the enzyme was incubated with glucose (Fig. 30A, Panel 2), MG (Fig. 30A Panel 3) or a combination of both glucose and MG (Fig. 30A, Panel 4), respectively, and 50 µg *A. vera* extract. The activity increased by 14.6, 10.1 and 9.8% as compared to the control when the enzyme was incubated with glucose

Fig. 28. Effect of TQ on the fluorescent AGEs formed of SOD due to glycation. AGEs specific fluorescence intensity at the excitation/emission wavelengths of 350/450 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM TQ. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).

Fig. 29. Effect of TQ on the fibrils formed in SOD due to glycation. ThT fluorescence intensity at the excitation/emission wavelengths of 440/480 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM TQ. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).


Fluorescence Intensity at 450 nm
20
15
10
5
0
1
2
3
4
Panel

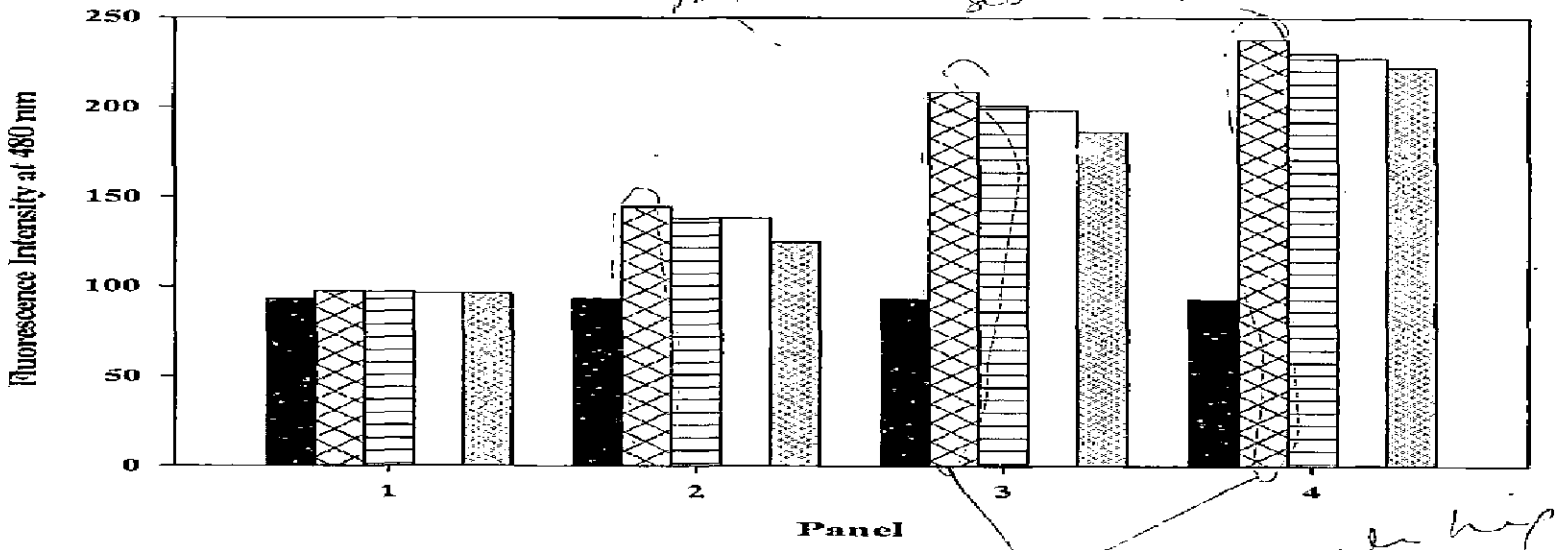
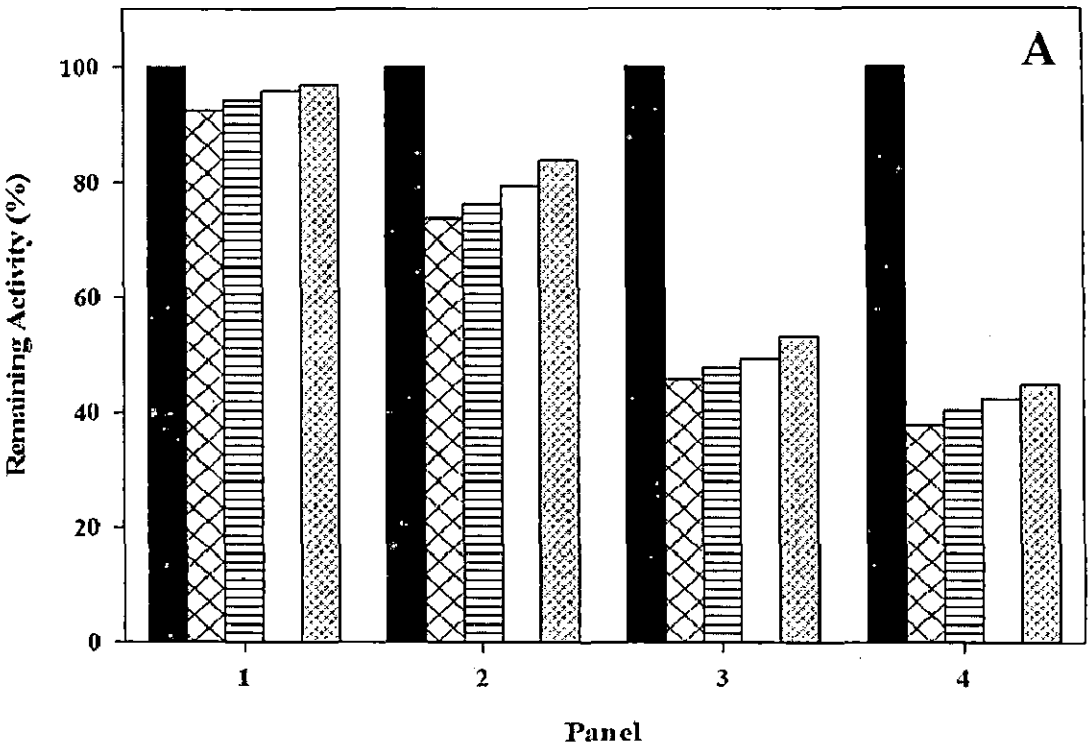
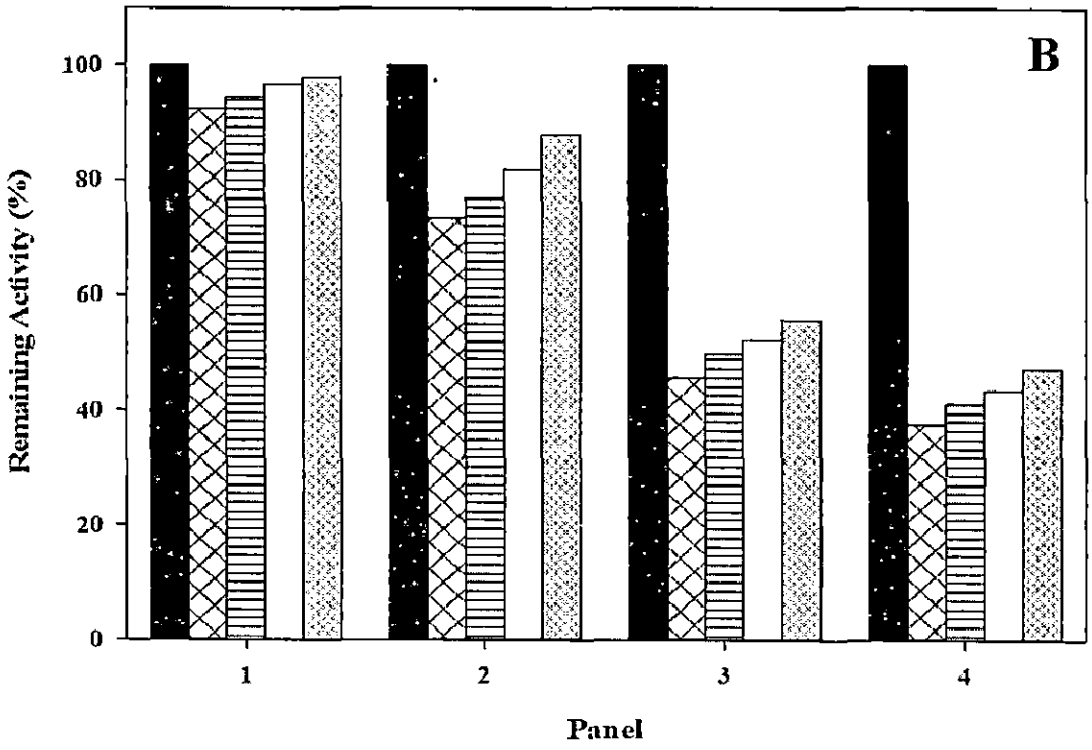

Fluorescence Intensity at 480 nm
250
200
150
100
50
0
1
2
3
4
Panel

Fig. 30. Effect of *A. vera* extract or aloin on the activity of SOD incubated with glucose, MG or both. SOD (0.2 mg/ml) was incubated alone (Panel 1), with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μg/ml of *A. vera* extract (A) or μM aloin (B). Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■). The enzyme activity was then determined under standard assay conditions. Each value represents the average for three independent experiments performed in duplicates.


A
Remaining Activity (%)
100
80
60
40
20
0
1
2
3
4
Panel


B
Remaining Activity (%)
100
80
60
40
20
0
1
2
3
4
Panel

(Fig. 30B, Panel 2), MG (Fig. 30B Panel 3) or a combination of both glucose and MG (fig. 30B Panel 4), respectively, and 50 μM aloin. This observed further increase in activity is believed to be due to the antiglycating activity of *A. vera* extract and aloin. The increase in activity was more in the case of glucose than for MG or both MG and glucose. Therefore, it appears that *A. vera* extract and aloin is a more effective antiglycating agent for sugars/compounds that are milder glycating agents.

4.4.2. SDS-PAGE

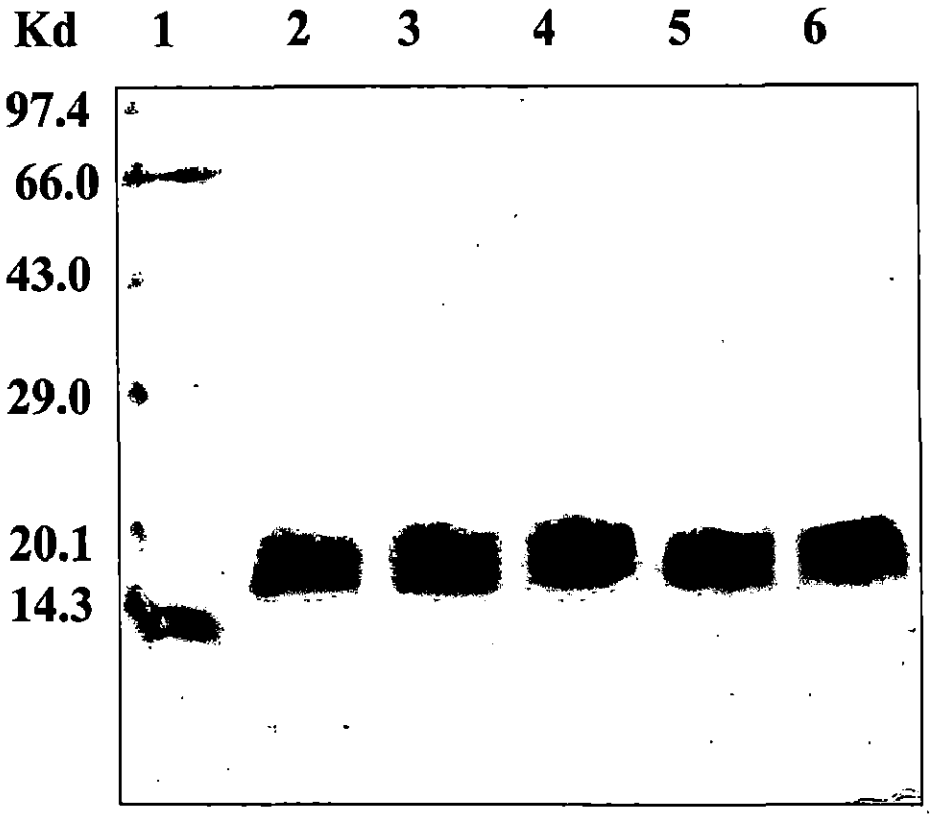
The protective effect of *A. vera* extract and aloin on SOD fragmentation/cross-linking induced by glycation is seen in Fig. 31 and 32, respectively. SDS-PAGE of SOD incubated for ten days in the absence of glucose or MG showed same staining intensity with increasing *A. vera* extract or aloin concentration (Fig. 31A and 32A, respectively). However, the enzyme incubated for ten days with glucose showed a more increase in staining intensity with increasing *A. vera* extract or aloin concentration (Fig. 31B and 32B, respectively). SOD incubated for ten days with MG (Fig. 31C and 32C) or a combination of both glucose and MG (Fig. 31D and 32D) exhibited a decrease in the bands corresponding to the cross-linked aggregates with increasing *A. vera* extract (Fig. 31C and 31D) or aloin (Fig. 32C and 32D) concentration. In fact in the case of MG alone, there was a slight increase in the band corresponding to the native enzyme with increasing *A. vera* extract or aloin concentration. Therefore, it is evident from SDS-PAGE analysis that *A. vera* extract and aloin protected SOD against fragmentation/cross-linking induced by glycation.

4.4.3. ELISA

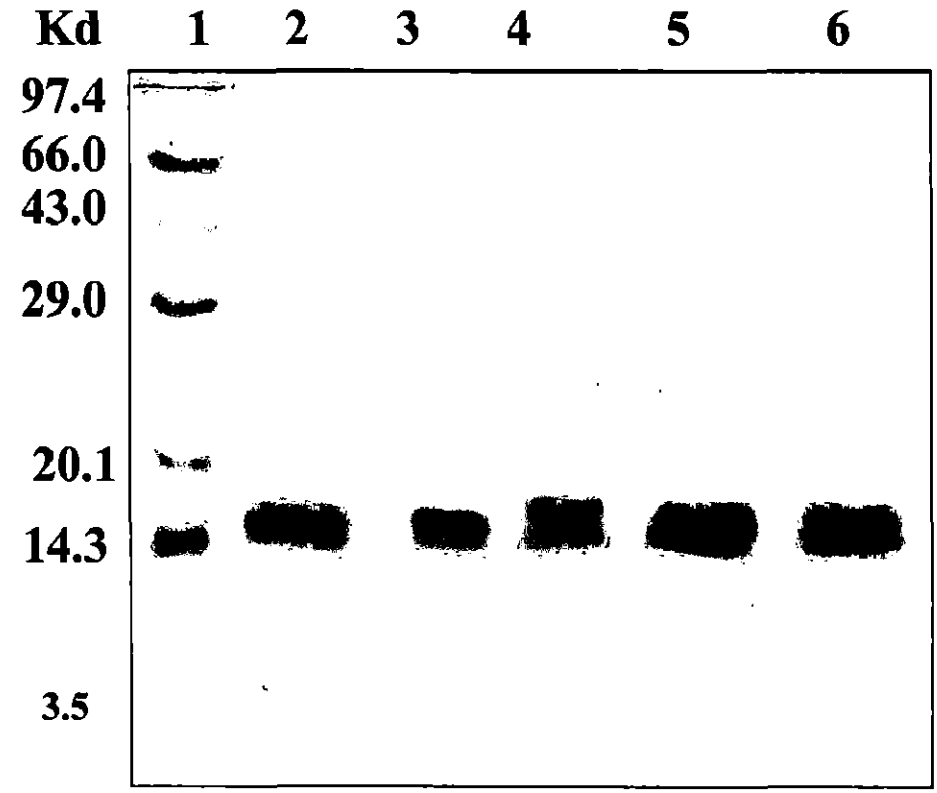
Incubation of SOD alone or with glucose, MG and both glucose and MG results in a decrease in absorbance at 490 nm in ELISA indicating reduced cross-reactivity with anti-SOD antibodies, which we believe is due to the structural/chemical modification of the epitopes of enzyme due to incubation at 37°C and by glycation. When SOD is incubated for 10 days at 37°C alone or with glucose, MG or both glucose and MG and increasing concentration of *A. vera* extract (Fig. 33A Panel 1, 2, 3 and 4, respectively) or aloin (Fig. 33B Panel 1, 2, 3 and 4, respectively), a progressive increase in absorbance at 490 nm (cross-reactivity with anti-SOD antibodies) with increasing *A. vera* extract or aloin concentration was observed in all the four cases. Therefore, this experiment shows that *A. vera* extract or

Fig. 31. SDS-PAGE of SOD incubated alone (A), with 0.5 M glucose (B), with 10 mM MG (C) or a combination of 0.5 M glucose and 10 mM MG (D) and with varying concentration of *A. vera* extract for 10 days at 37°C. Lane 1 shows molecular weight markers (Genei); Lane 2 shows SOD (10 μg) that has not been incubated with glucose, MG or *A. vera* extract. Lanes 3, 4, 5 and 6 show SOD (10 μg) incubated for 10 days alone or with glucose, MG or a combination of glucose and MG and with 0, 10, 20 and 50 μg/ml *A. vera* extract, respectively

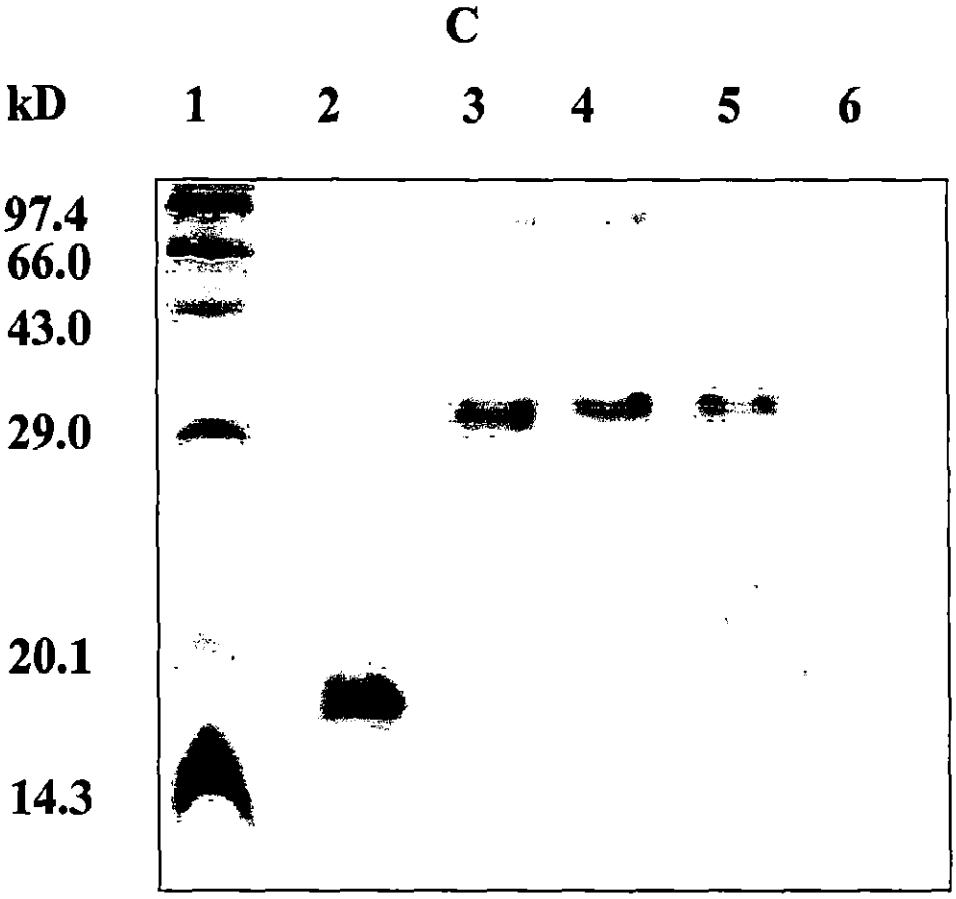
A


Kd
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3

B


Kd
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3
3.5

C


kD
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3

D

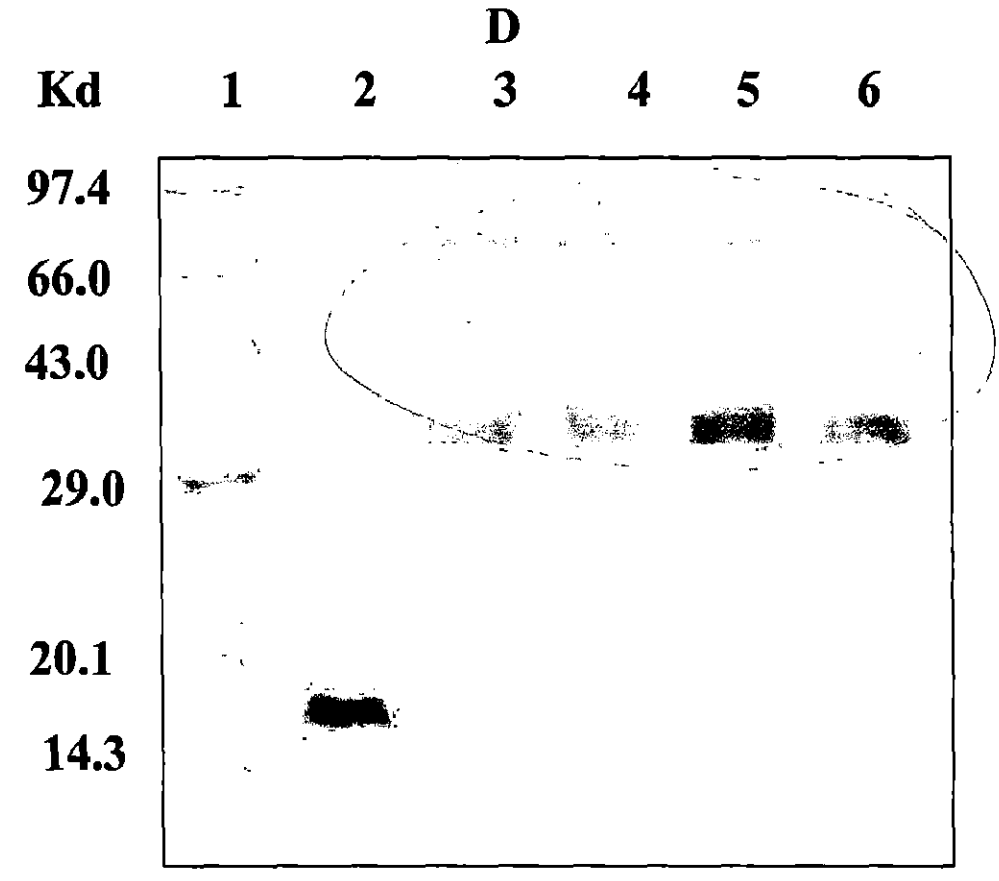
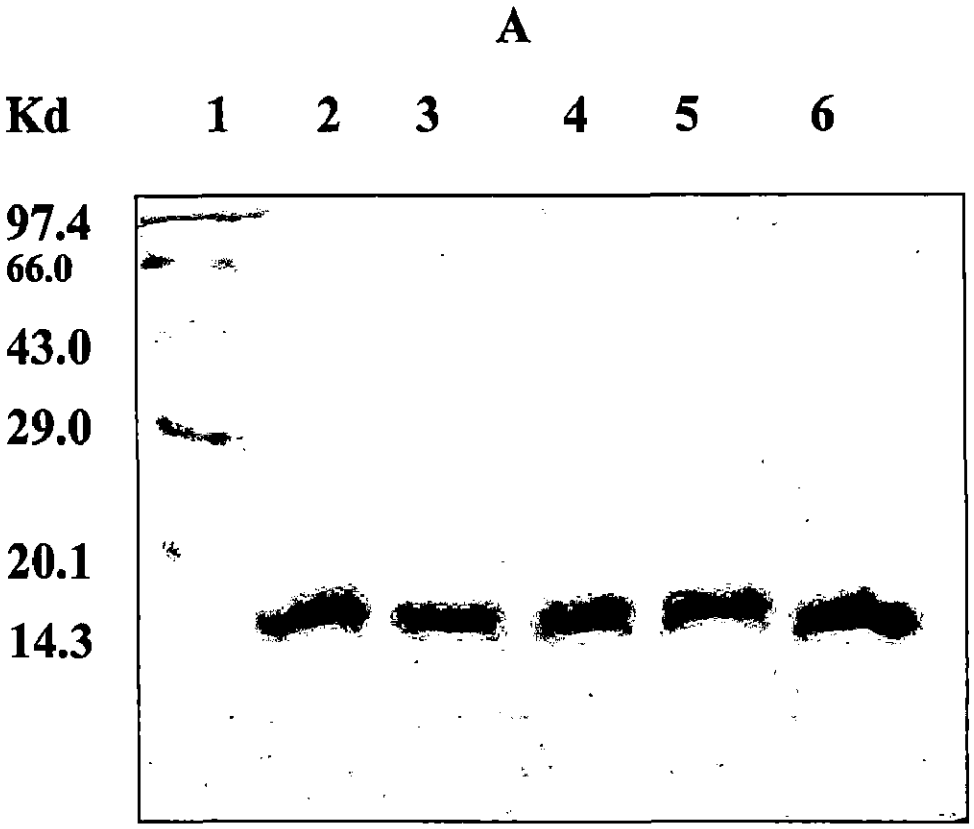
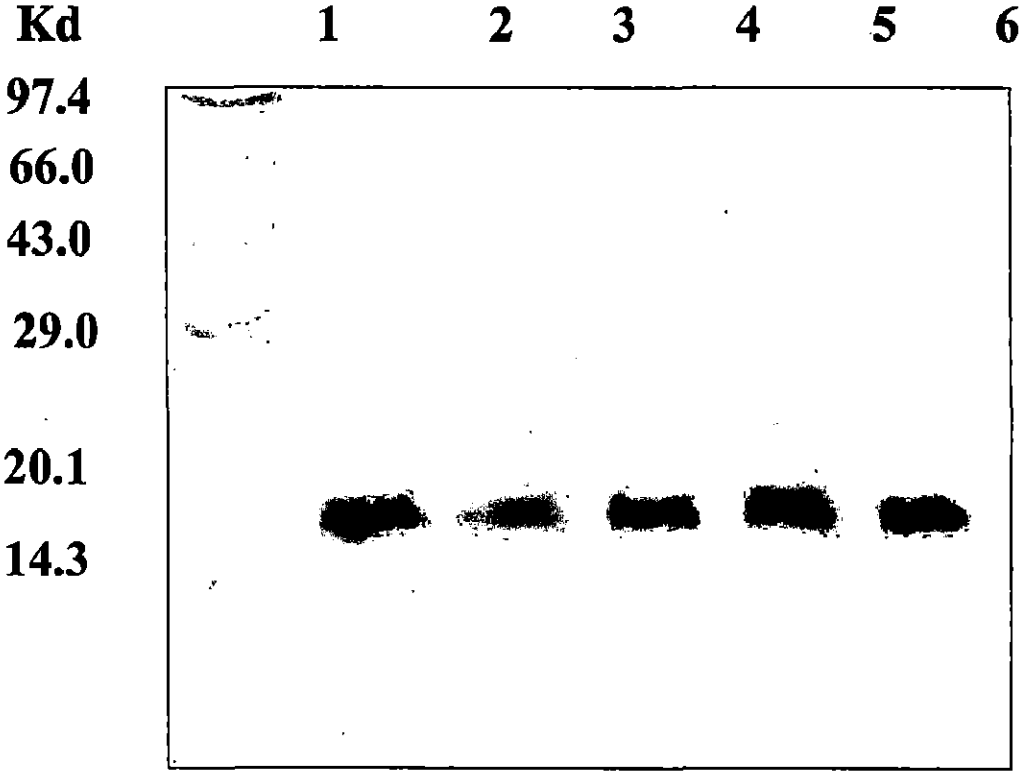

Kd
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3

Fig. 32. SDS-PAGE of SOD incubated alone (A), with 0.5 M glucose (B), with 10 mM MG (C) or a combination of 0.5 M glucose and 10 mM MG (D) and with varying concentration of aloin for 10 days at 37°C. Lane 1 shows molecular weight markers (Genei); Lane 2 shows SOD (10 μg) that has not been incubated with glucose, MG or aloin. Lanes 3, 4, 5 and 6 show SOD (10 μg) incubated for 10 days alone or with glucose, MG or a combination of glucose and MG and with 0, 10, 20 and 50 μM aloin, respectively.

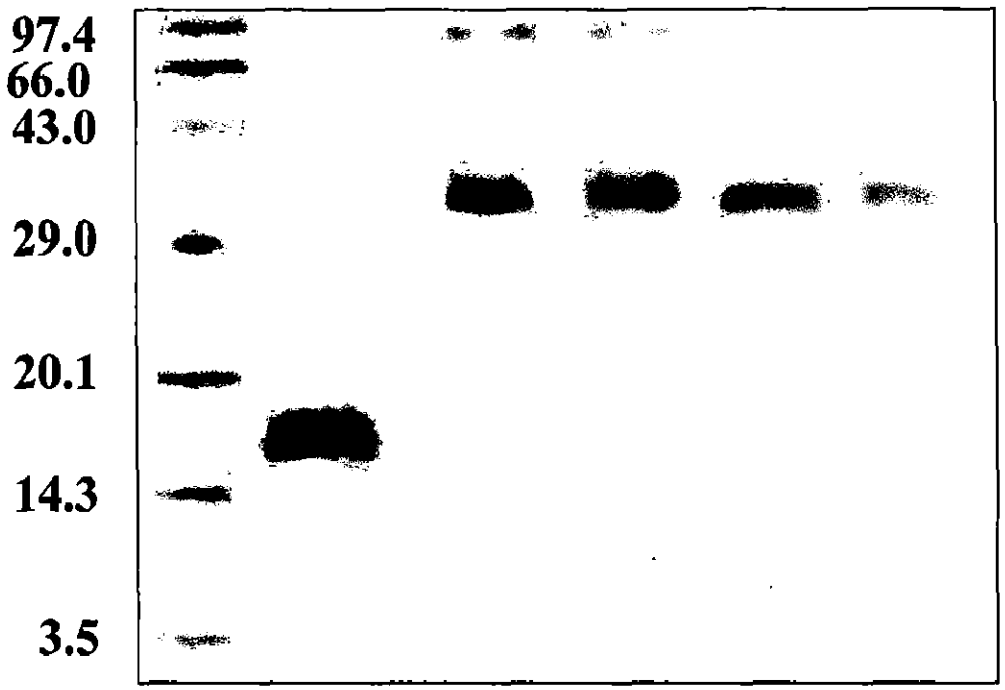
A


Kd
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3

B


Kd
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3

C


kD
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3
3.5

D

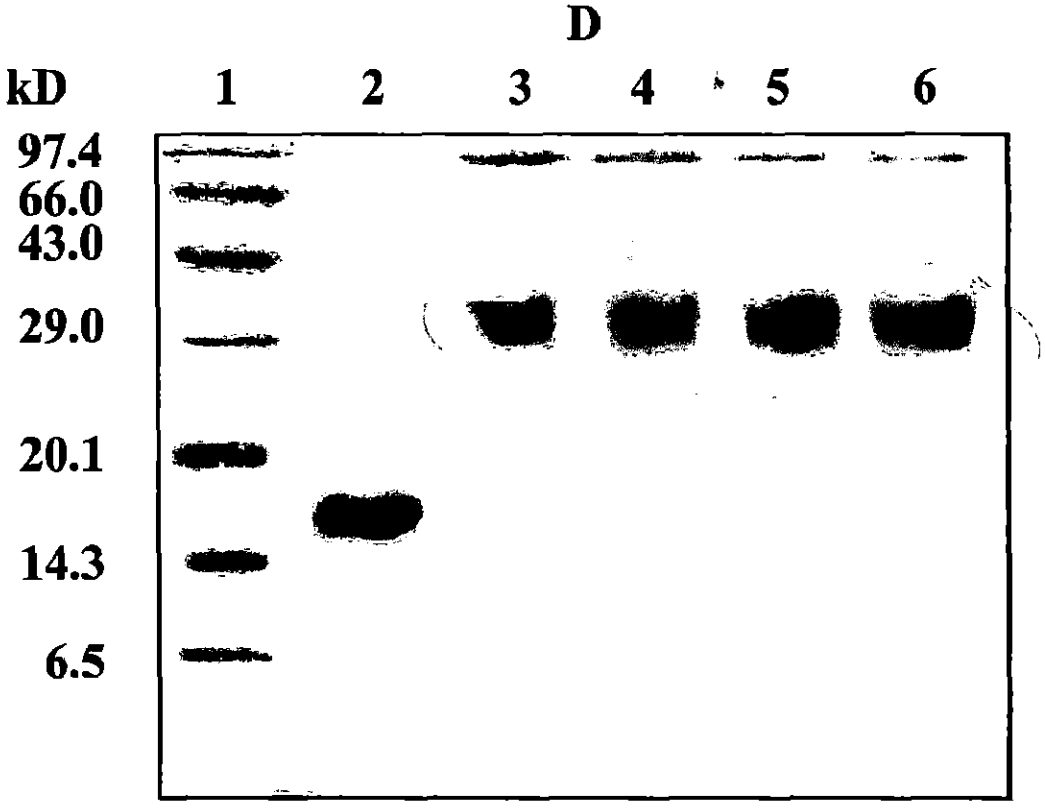
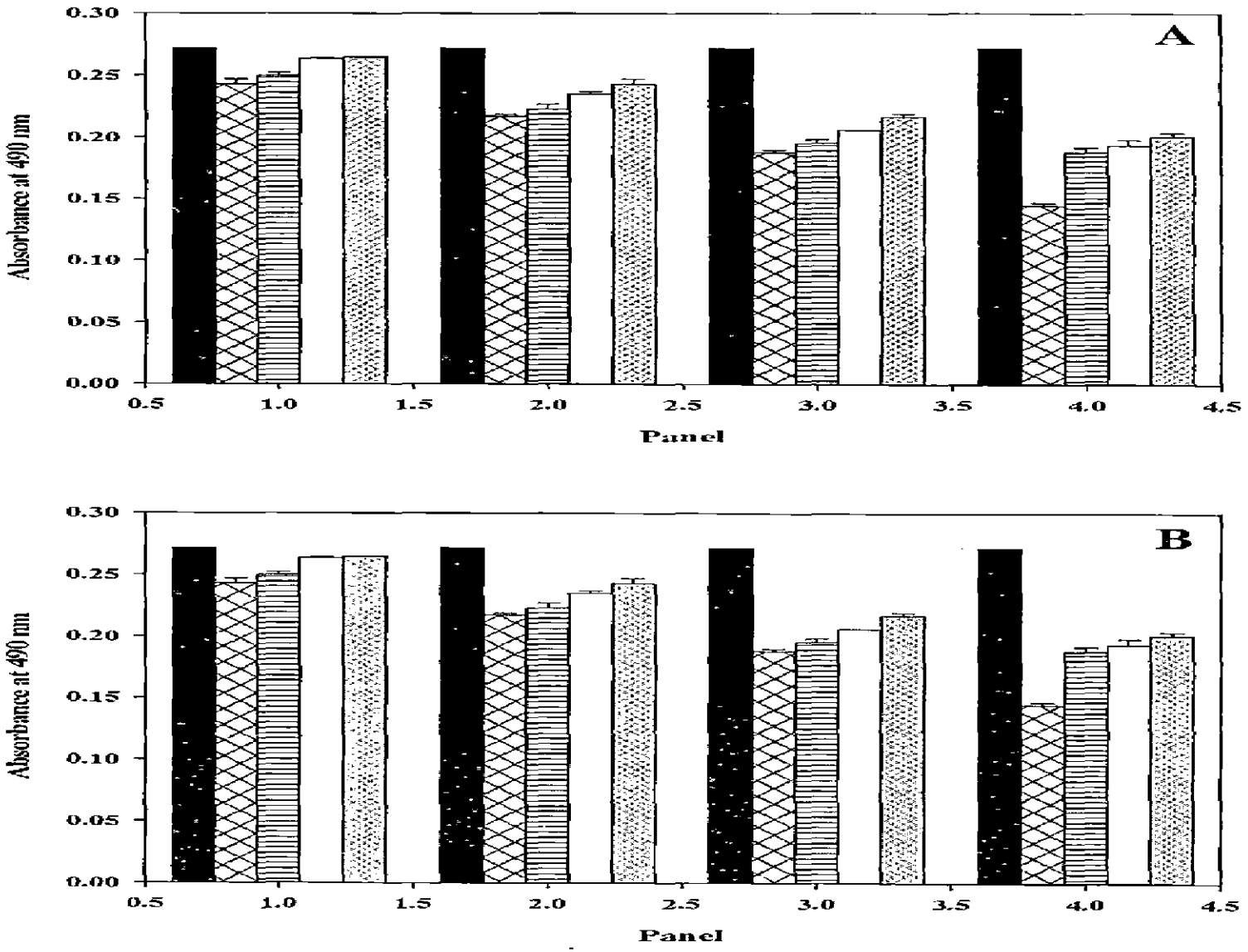

kD
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3
6.5

Fig. 33. Effect of *A. vera* extract or aloin on the cross-reactivity (ELISA) of anti-SOD antibodies with SOD incubated with glucose, MG or both. SOD (0.2 mg/ml) was incubated alone (Panel 1), with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μg/ml of *A. vera* extract (A) or μM aloin (B). Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■). Each value represents the average for two independent experiments performed in triplicates.


A
B
Absorbance at 490 nm
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Panel

aloin protected the enzyme to some extent against the structural/chemical changes induced by incubation at 37°C and glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of *A. vera* extract or aloin.

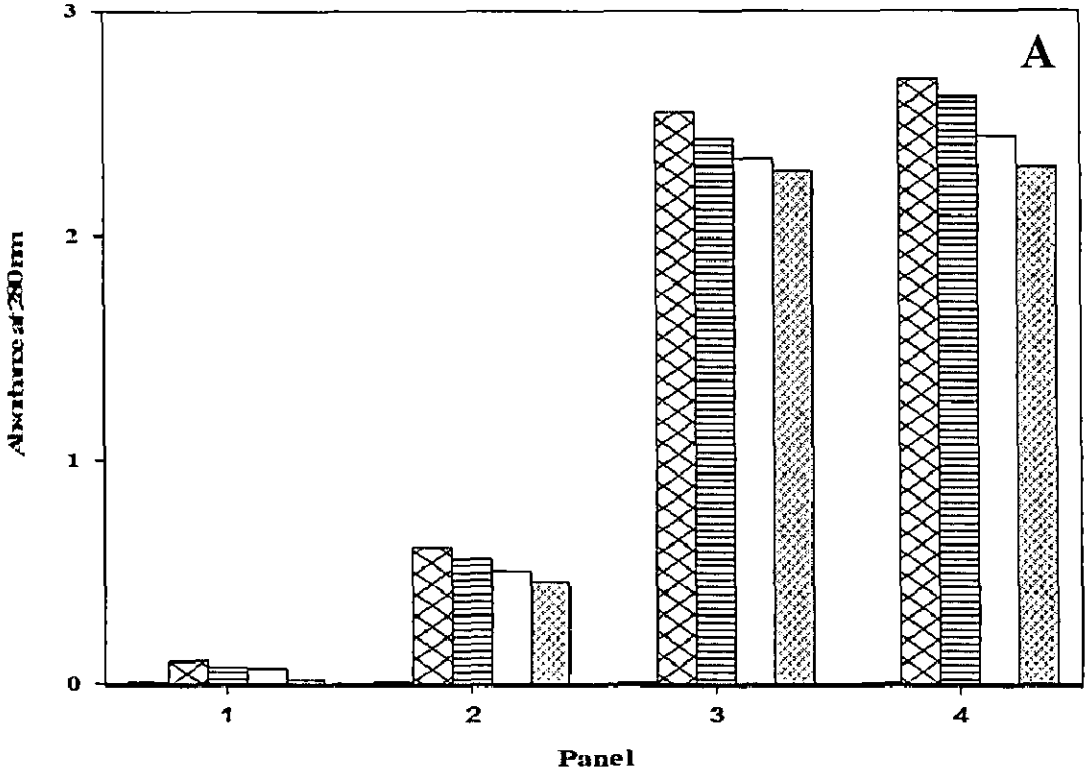
4.4.4. UV absorption studies

When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of *A. vera* extract or aloin, a progressive decrease in absorbance at 280 nm with increasing *A. vera* extract (Fig. 34A, Panel 2, 3 and 4, respectively) or aloin (Fig. 34B, Panel 2, 3 and 4, respectively) concentration was observed in all the three cases. SOD incubated alone for 10 days with increasing *A. vera* extract (Fig. 34A, Panel 1) or aloin (Fig. 34B, Panel 1) concentration (control) exhibited very slight decrease in absorbance at 280 nm. Therefore, *A. vera* extract or aloin protected the enzyme to some extent against the structural changes induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of *A. vera* extract or aloin. However, the enzyme was still far from the structure of the native enzyme even at 50 µg or 50 µM concentration of *A. vera* extract or aloin, respectively, in all the three cases.

4.4.5. Intrinsic fluorescence studies

When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of *A. vera* extract (Fig. 35A, Panel 2, 3 and 4, respectively) or aloin (Fig. 35B, Panel 2, 3 and 4, respectively), a progressive increase in fluorescence at 310 nm with increasing *A. vera* extract or aloin concentration was observed in all the three cases. The control exhibited insignificant increase in fluorescence at 310 nm (Fig. 35A and 35B, Panel 1). Therefore, again this experiment shows that *A. vera* extract or aloin protected the enzyme to some extent against the structural changes induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of *A. vera* extract or aloin. However, it was observed that SOD incubated for 10 days at 37°C with glucose, without *A. vera* extract or aloin but with 1% DMSO showed fluorescence enhancement at 310 nm and not quenching (Fig. 35A and 35B, Panel 2 Column 2). Therefore, it appears that in the samples of SOD glycated by glucose in the presence of DMSO, the environment around the aromatic residues of the protein is somehow perturbed which affects their fluorescence.

Fig. 34. Effect of *A. vera* extract or aloin on the absorption changes induced in SOD due to glycation. Absorbance at 280 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μg/ml of *A. vera* extract (A) or μM aloin (B). Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).



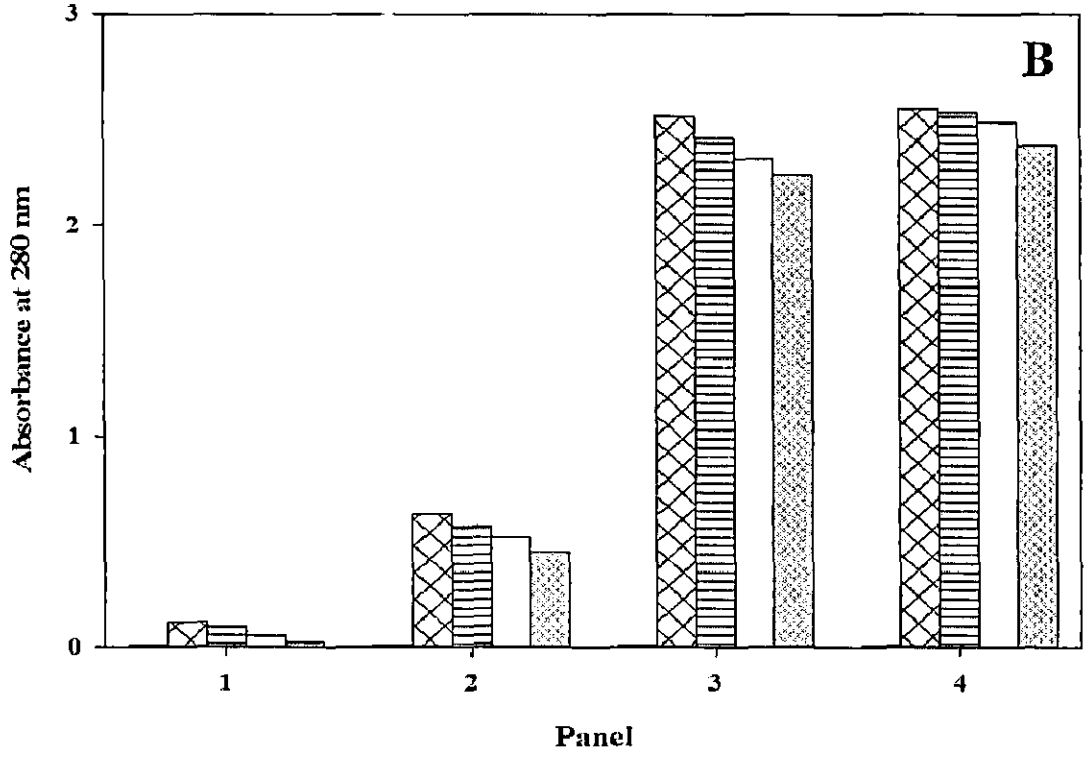
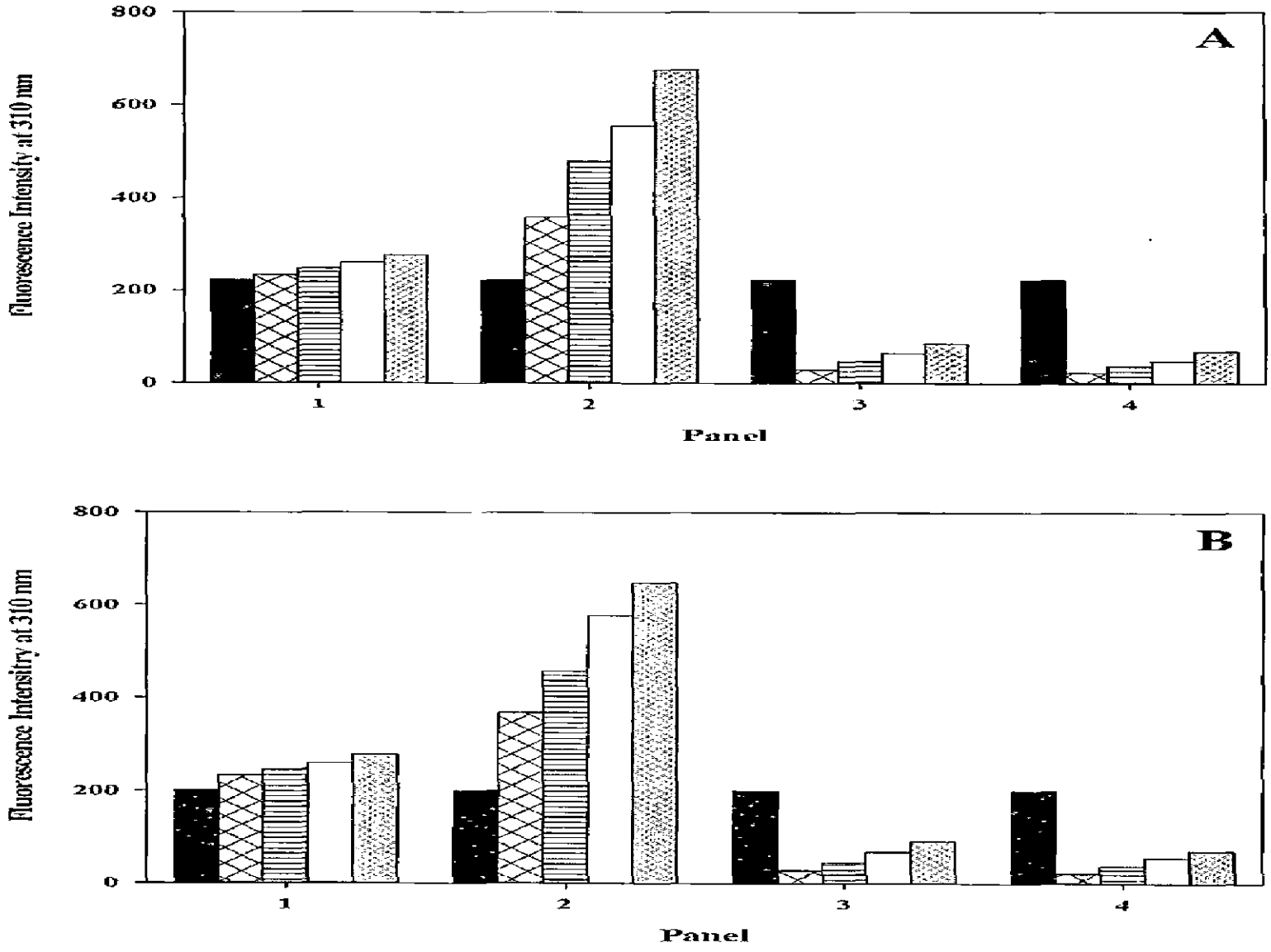


Fig. 35. Effect of *A. vera* extract or aloin on the intrinsic fluorescence changes induced in SOD due to glycation. Fluorescence intensity at the excitation/emission wavelengths of 280/310 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μg/ml of *A. vera* extract (A) or μM aloin (B). Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).


A
B
800
600
400
200
0
Fluorescence Intensity at 310 nm
1
2
3
4
Panel

4.4.6. AGEs specific fluorescence studies

When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of *A. vera* extract (Fig. 36A, Panel 2, 3 and 4, respectively) or aloin (Fig. 36B, Panel 2, 3 and 4, respectively), a progressive decrease in AGEs specific fluorescence at 450 nm with increasing *A. vera* extract or aloin concentration was observed in all the three cases. The control exhibited insignificant decrease in fluorescence at 450 nm (Fig. 36A and 36B, Panel 1). Therefore, *A. vera* extract or aloin protected the enzyme to some extent against formation of AGEs induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of *A. vera* extract or aloin.

4.4.7. ThT fluorescence studies

Glycation of SOD by glucose, MG and both glucose and MG results in ThT fluorescence enhancement at 480 nm (formation of fibrils). When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of *A. vera* extract (Fig. 37A, Panel 2, 3 and 4, respectively) or aloin (Fig. 37B, Panel 2, 3 and 4, respectively), a progressive decrease in ThT fluorescence with increasing *A. vera* extract or aloin concentration was observed in all the three cases. The control exhibited insignificant decrease in ThT fluorescence at 480 nm (Fig. 37A and 37B Panel 1). Therefore, *A. vera* extract or aloin protected the enzyme to some extent against formation of fibrils induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of *A. vera* extract or aloin.

4.5. Protective effect of EA on the glycation of SOD with glucose or MG

4.5.1. Activity studies

The effect of EA on the activity of SOD glycated by 0.5 M glucose or 10 mM MG was studied. Fig. 38 shows the percentage remaining activity of SOD incubated for ten days at 37°C alone, and that incubated in the presence of glucose, MG or a combination of both and with increasing concentration of EA. SOD incubated for ten days alone with EA showed a slight increase in activity with increasing EA concentration (Fig. 38, Panel 1). The activity increased by 2.8% when the enzyme was incubated with 50 μM EA respectively as compared to the control (the sample

Fig. 36. Effect of *A. vera* extract or aloin on the fluorescent AGEs formed of SOD due to glycation. AGEs specific fluorescence intensity at the excitation/emission wavelengths of 350/450 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μg/ml of *A. vera* extract (A) or μM aloin (B). Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).

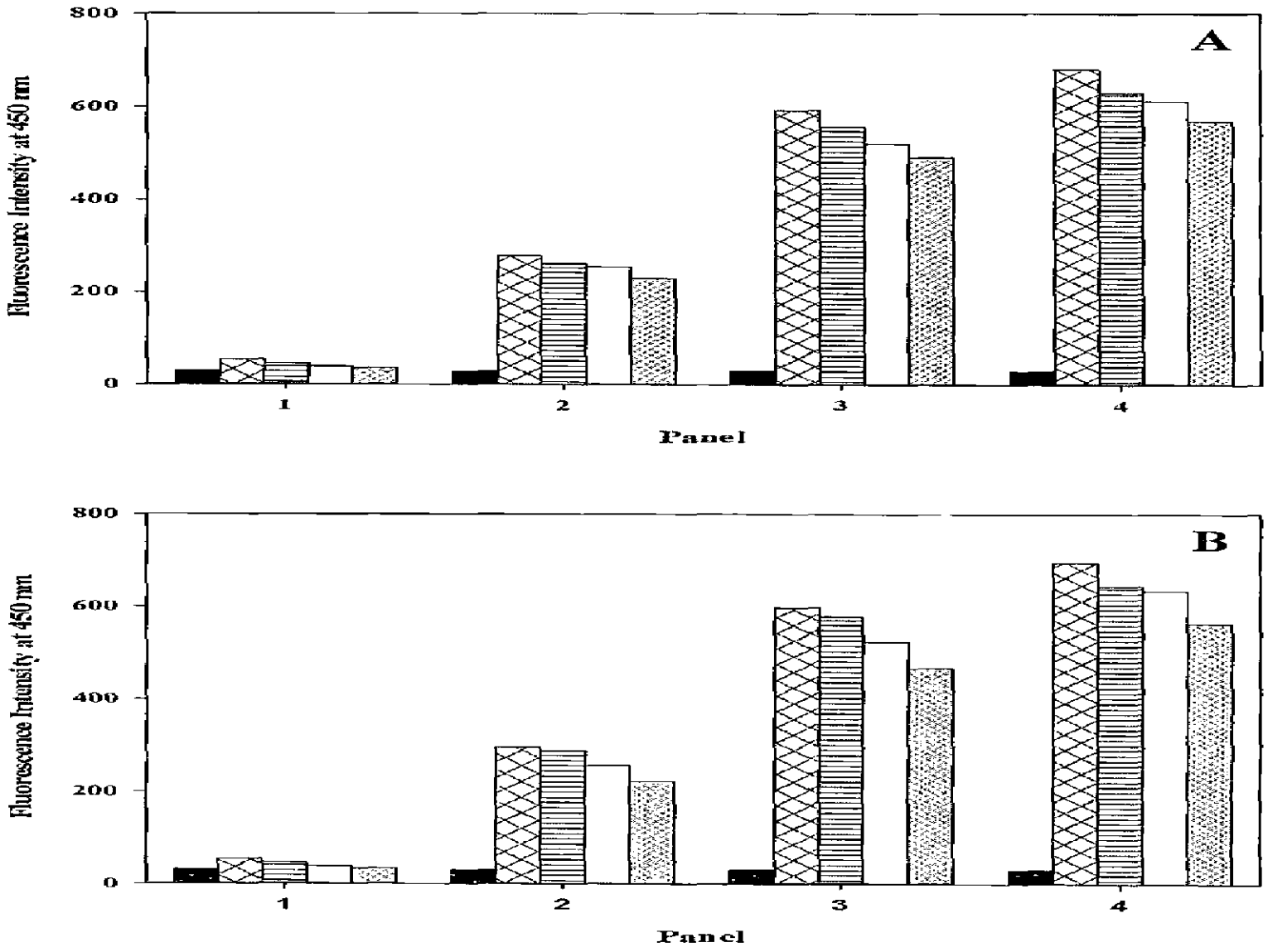

A
Fluorescence Intensity at 450 nm
800
600
400
200
0
1
2
3
4
Panel
B
Fluorescence Intensity at 450 nm
800
600
400
200
0
1
2
3
4
Panel

Fig. 37. Effect of *A. vera* extract or aloin on the fibrils formed in SOD due to glycation. ThT fluorescence intensity at the excitation/emission wavelengths of 440/480 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μg/ml of *A. vera* extract (A) or μM aloin (B). Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).

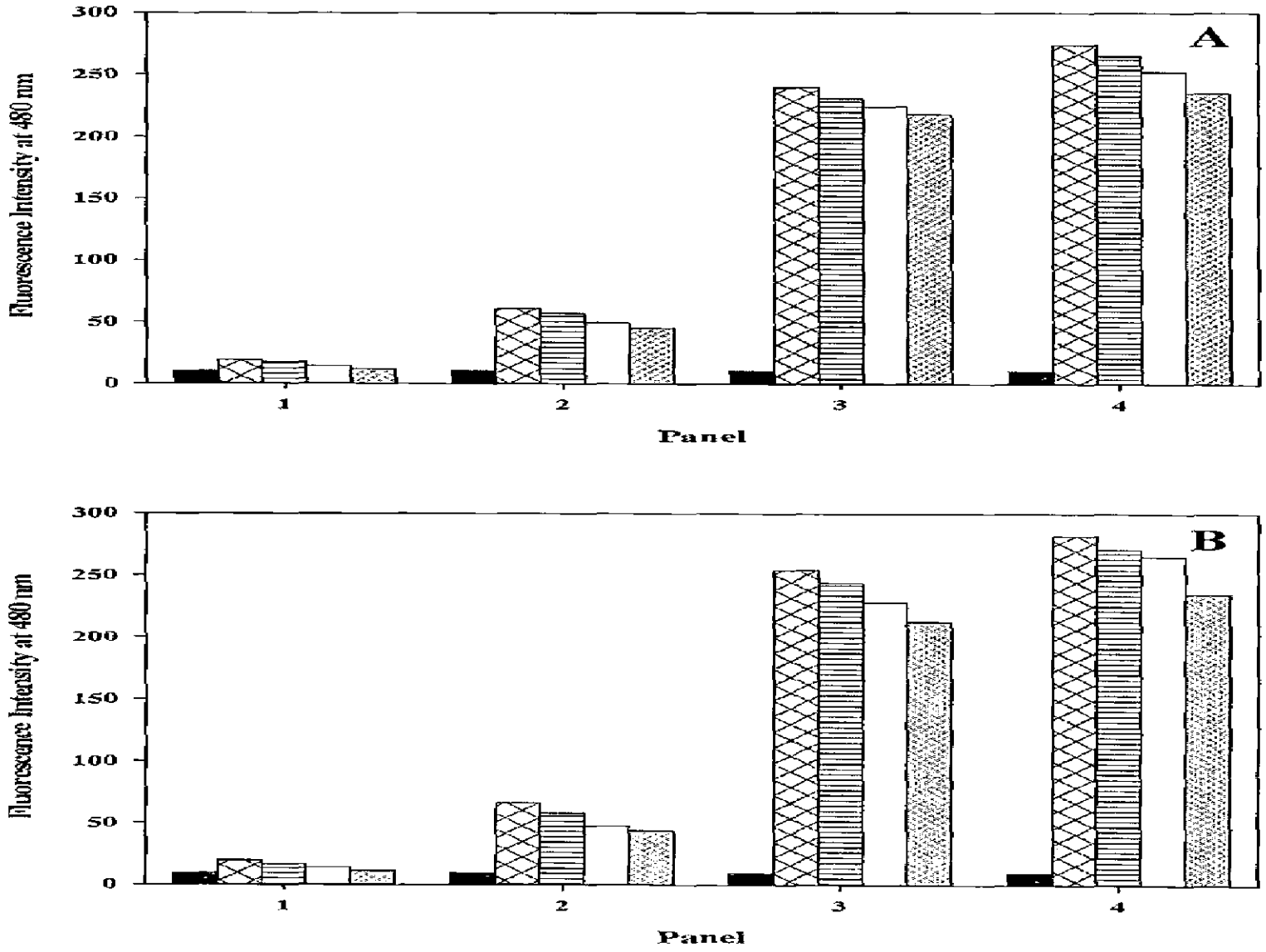
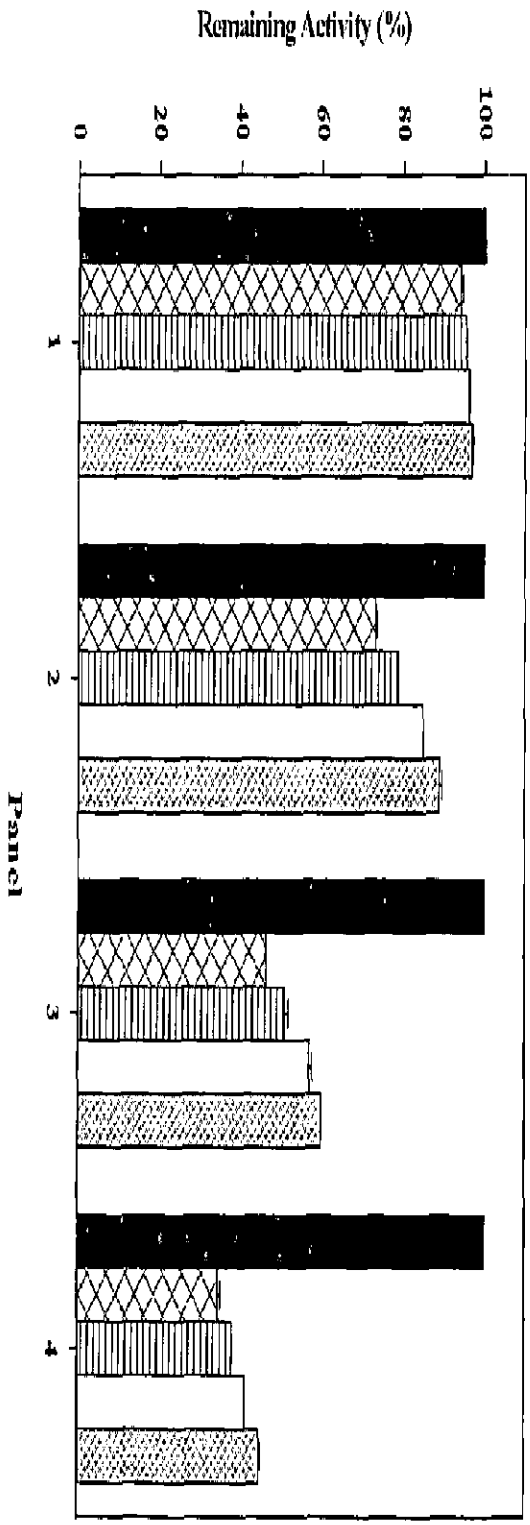

A
B
Fluorescence Intensity at 480 nm
300
250
200
150
100
50
0
1
2
3
4
Panel

Fig. 38. Effect of EA on the activity of SOD incubated with glucose, MG or both. SOD (0.2 mg/ml) was incubated alone (Panel 1), with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM EA. Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■). The enzyme activity was then determined under standard assay conditions. Each value represents the average for three independent experiments performed in duplicates.


Remaining Activity (%)
0
20
40
60
80
100
Panel
1
2
3
4

that had no EA). This slight increase in activity of SOD is believed to be due the antioxidant property of EA. SOD incubated with glucose, MG or a combination of both, and EA showed a greater increase in activity as compared to the enzyme that was not incubated with glucose or MG. The activity increased by 15.9, 13.7 and 10.0% as compared to the control when the enzyme was incubated with glucose (Fig. 38, Panel 2), MG (Fig. 38, Panel 3) or a combination of both glucose and MG (Fig. 38, Panel 4), respectively, and 50 μM EA. This observed further increase in activity is believed to be due to the antiglycating activity of EA. The data clearly indicates that the increase in activity was more in the case of glucose than for MG or both MG and glucose. Therefore, EA is a more effective antiglycating agent for sugars/compounds that are milder glycating agents.

4.5.2. SDS-PAGE

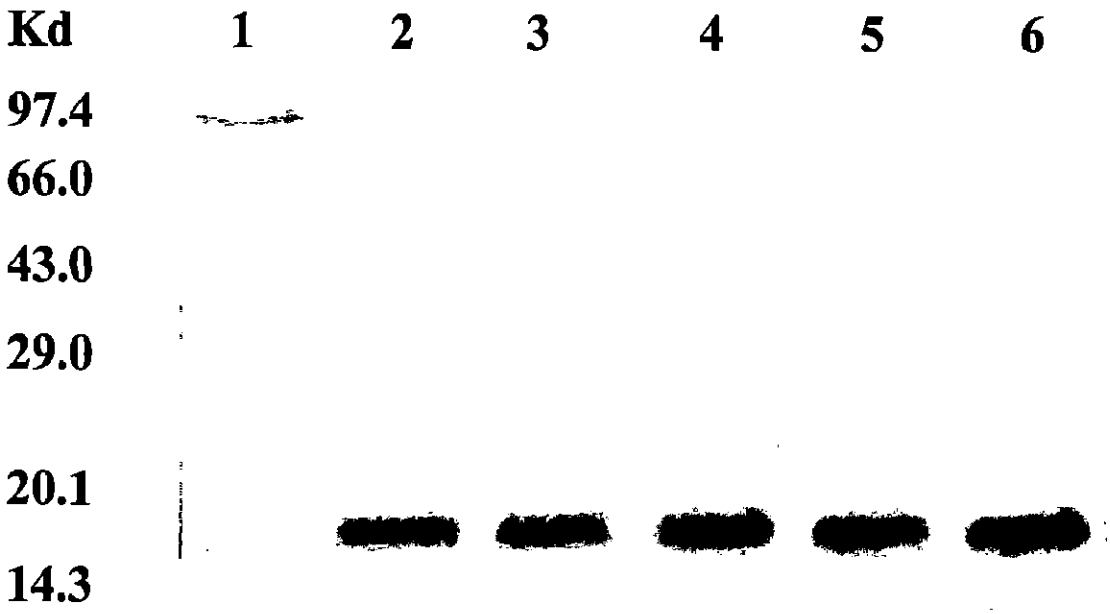
The protective effect of EA on SOD fragmentation/cross-linking induced by glycation is seen in fig. 39. SDS-PAGE of SOD incubated for ten days in the absence of glucose or MG showed same staining intensity with increasing EA concentration (Fig. 39A). However, the enzyme incubated for ten days with glucose showed a more increase in staining intensity with increasing EA concentration (Fig. 39B). SOD incubated for ten days with MG (Fig. 39C) or a combination of both glucose and MG (Fig. 39D) exhibited a decrease in the bands corresponding to the cross-linked aggregates with increasing EA concentration. Infact in the case of MG alone, there was a slight increase in the band corresponding to the native enzyme with increasing EA concentration. Therefore, it is evident from SDS-PAGE analysis that EA protected SOD against fragmentation/cross-linking induced by glycation.

4.5.3. ELISA

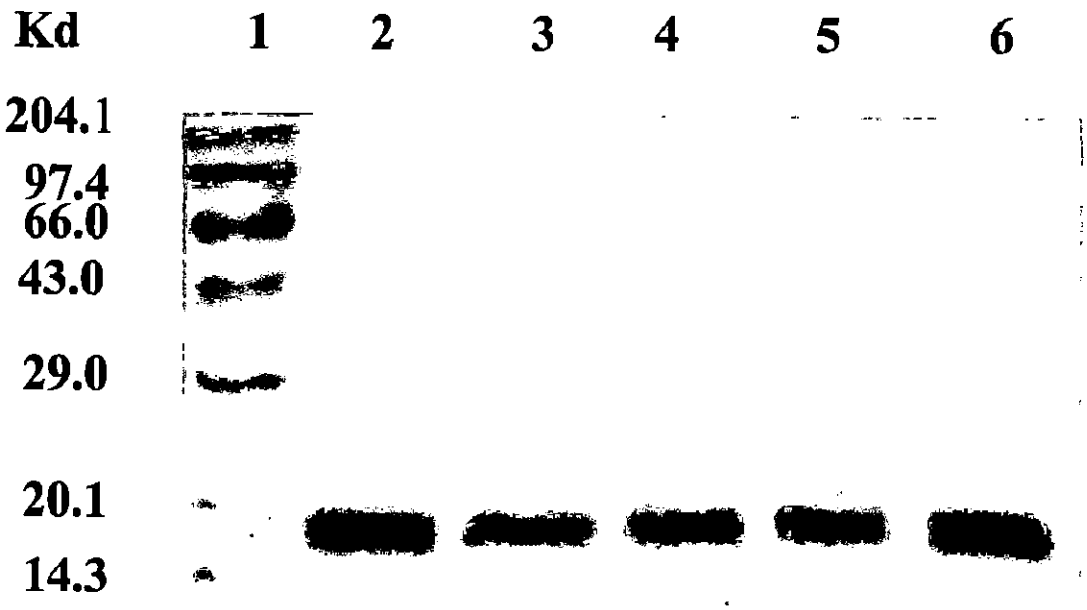
Incubation of SOD alone or with glucose, MG and both glucose and MG results in a decrease in absorbance at 490 nm in ELISA indicating reduced cross-reactivity with anti-SOD antibodies, which we believe is due to the structural/chemical modification of the epitopes of enzyme due to incubation at 37°C and by glycation. When SOD is incubated for 10 days at 37°C alone or with glucose, MG or both glucose and MG and increasing concentration of EA, a progressive increase in absorbance at 490 nm (cross-reactivity with anti-SOD antibodies) with increasing EA concentration (Fig. 40) was observed in all the four cases. Therefore,

Fig. 39. SDS-PAGE of SOD incubated alone (A), with 0.5 M glucose (B), with 10 mM MG (C) or a combination of 0.5 M glucose and 10 mM MG (D) and with varying concentration of EA for 10 days at 37°C. Lane 1 shows molecular weight markers (Genei); Lane 2 shows SOD (10 μg) that has not been incubated with glucose, MG or EA. Lanes 3, 4, 5 and 6 show SOD (10 μg) incubated for 10 days alone or with glucose, MG or a combination of glucose and MG and with 0, 10, 20 and 50 μM EA, respectively.

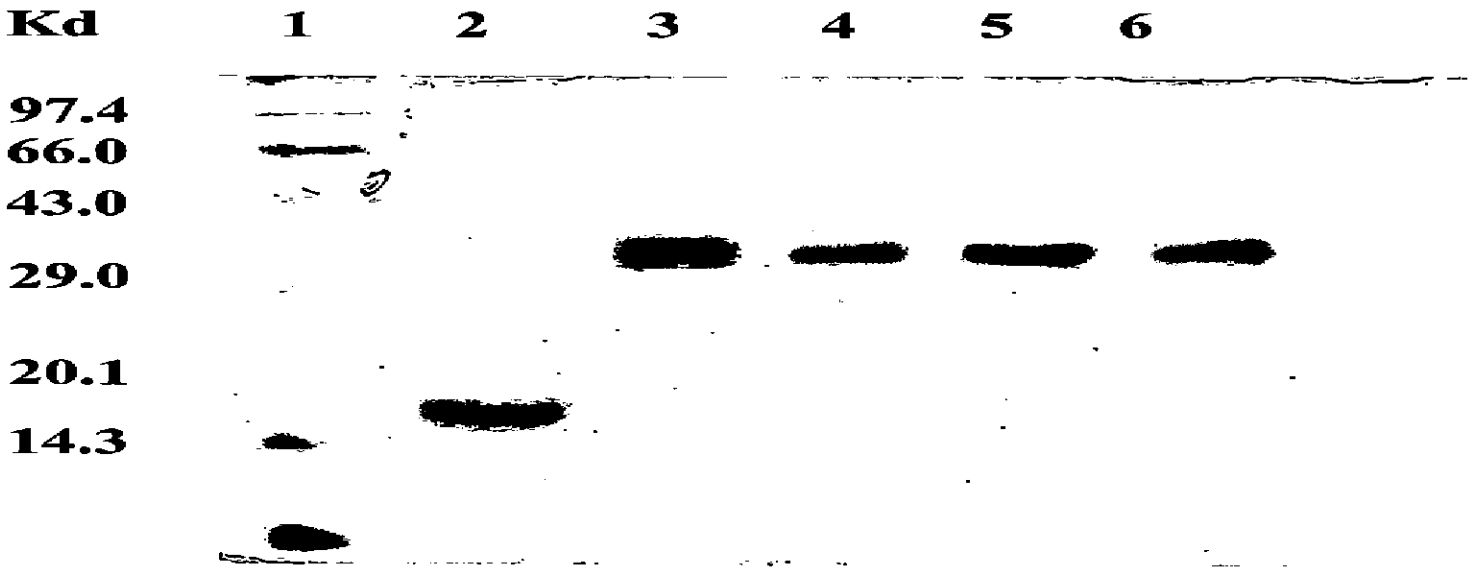
A


Kd
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3

B


Kd
1
2
3
4
5
6
204.1
97.4
66.0
43.0
29.0
20.1
14.3

C


Kd
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3

D

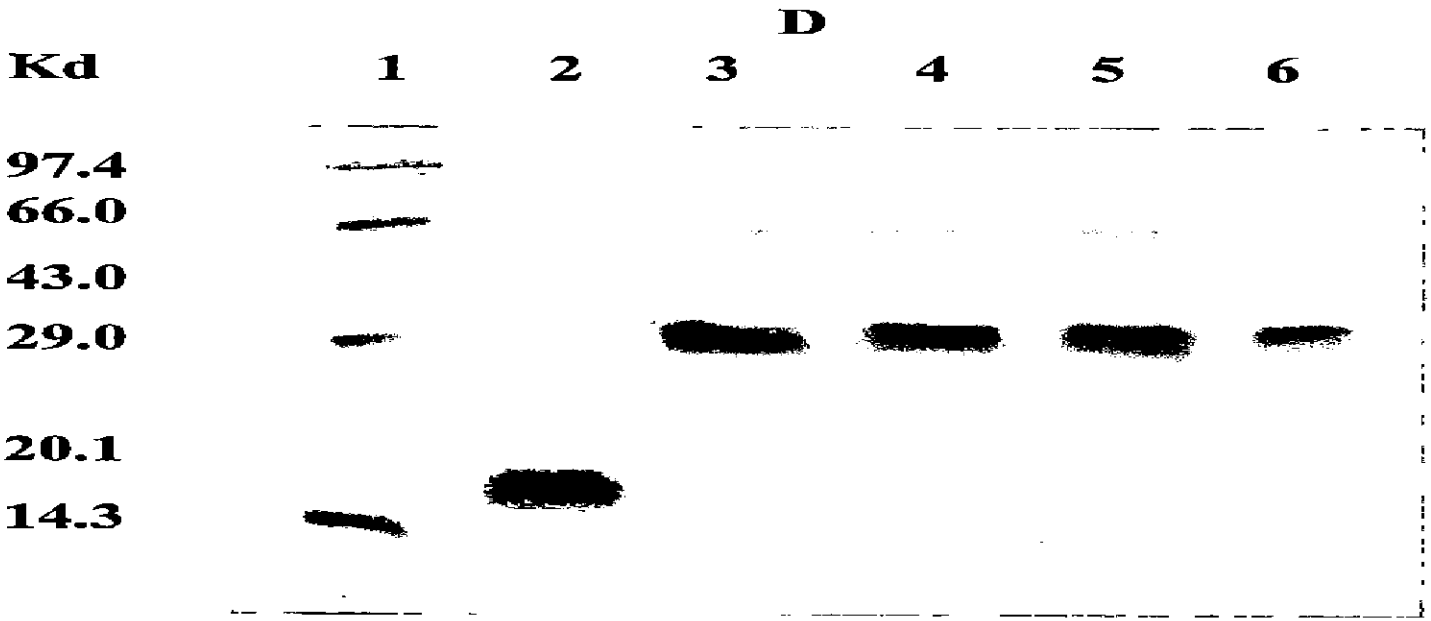
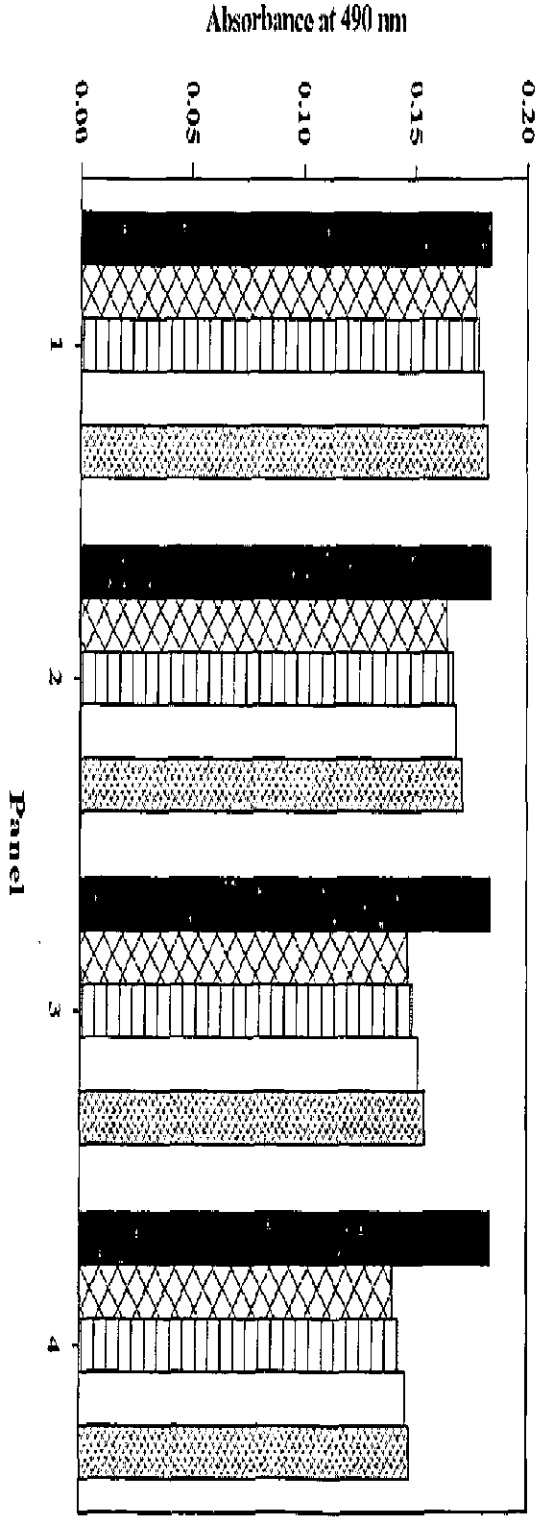

Kd
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3

Fig. 40. Effect of EA on the cross-reactivity (ELISA) of anti-SOD antibodies with SOD incubated with glucose, MG or both. SOD (0.2 mg/ml) was incubated alone (Panel 1), with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM EA. Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■). Each value represents the average for two independent experiments performed in triplicates.


Absorbance at 490 nm
0.00
0.05
0.10
0.15
0.20
Panel
1
2
3
4

this experiment shows that EA protected the enzyme to some extent against the structural/chemical changes induced by incubation at 37°C and glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of EA.

4.5.4. UV absorption studies

When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of EA, a progressive decrease in absorbance at 280 nm with increasing EA (Fig. 41, Panel 2, 3 and 4, respectively) was observed in all the three cases. SOD incubated alone for 10 days with increasing EA concentration (control) exhibited very slight decrease in absorbance at 280 nm (Fig. 41, Panel 1). Therefore, EA protected the enzyme to some extent against the structural changes induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of EA. However, the enzyme was still far from the structure of the native enzyme even at 50 μM concentration of EA, respectively, in all the three cases.

4.5.5. Intrinsic fluorescence studies

When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of EA (Fig. 42, Panel 2, 3 and 4, respectively), a progressive increase in fluorescence at 310 nm with increasing EA concentration was observed in all the three cases. The control exhibited insignificant increase in fluorescence at 310 nm (Fig. 42, Panel 1). Therefore, again this experiment shows that EA protected the enzyme to some extent against the structural changes induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of EA. However, it was observed that SOD incubated for 10 days at 37°C with glucose, without EA but with 1% DMSO showed fluorescence enhancement at 310 nm and not quenching (Fig. 42, Panel 2 Column 2). Therefore, it appears that in the samples of SOD glycated by glucose in the presence of DMSO, the environment around the aromatic residues of the protein is somehow perturbed which affects their fluorescence.

Fig. 41. Effect of EA on the absorption changes induced in SOD due to glycation. Absorbance at 280 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM EA. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).

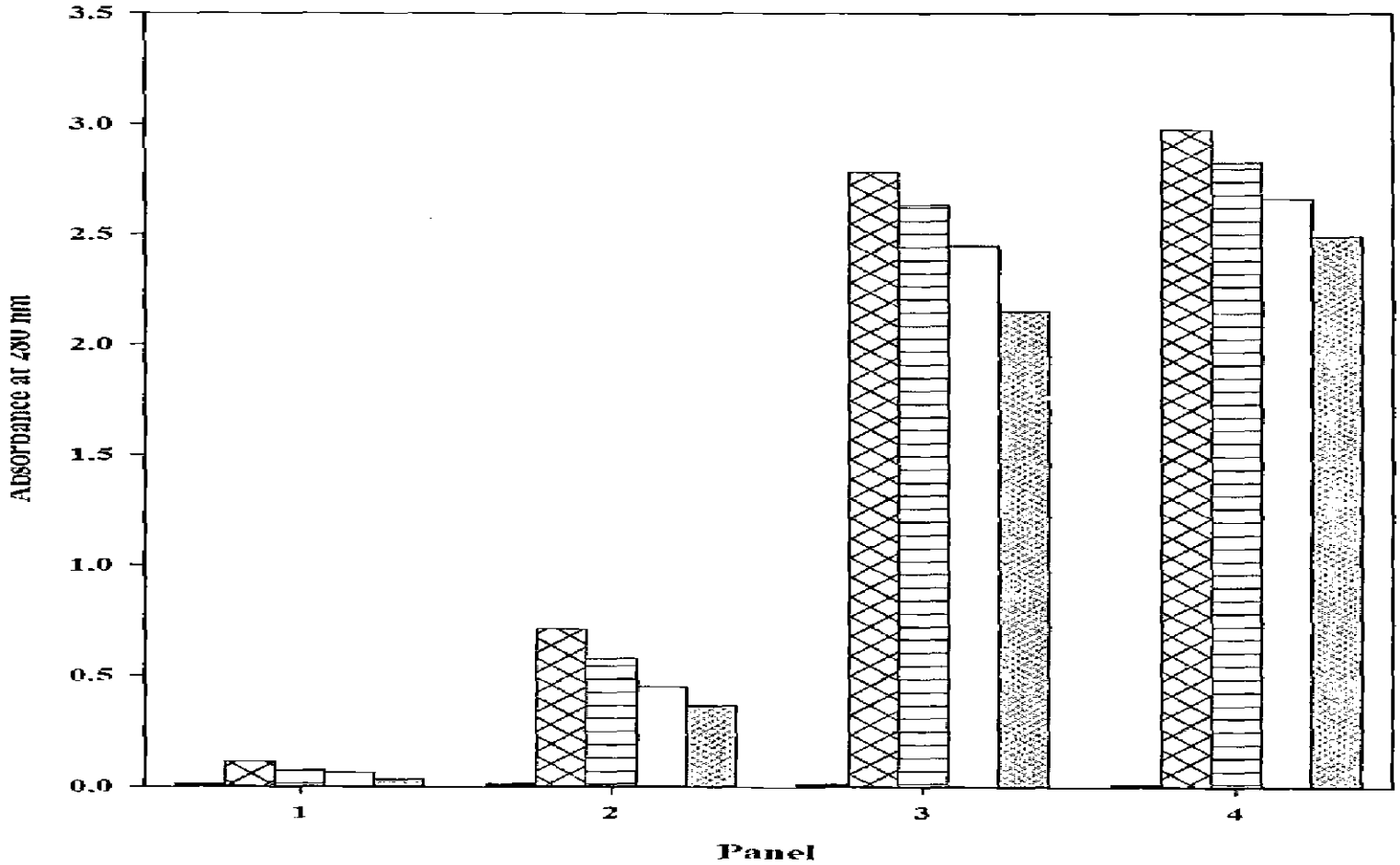
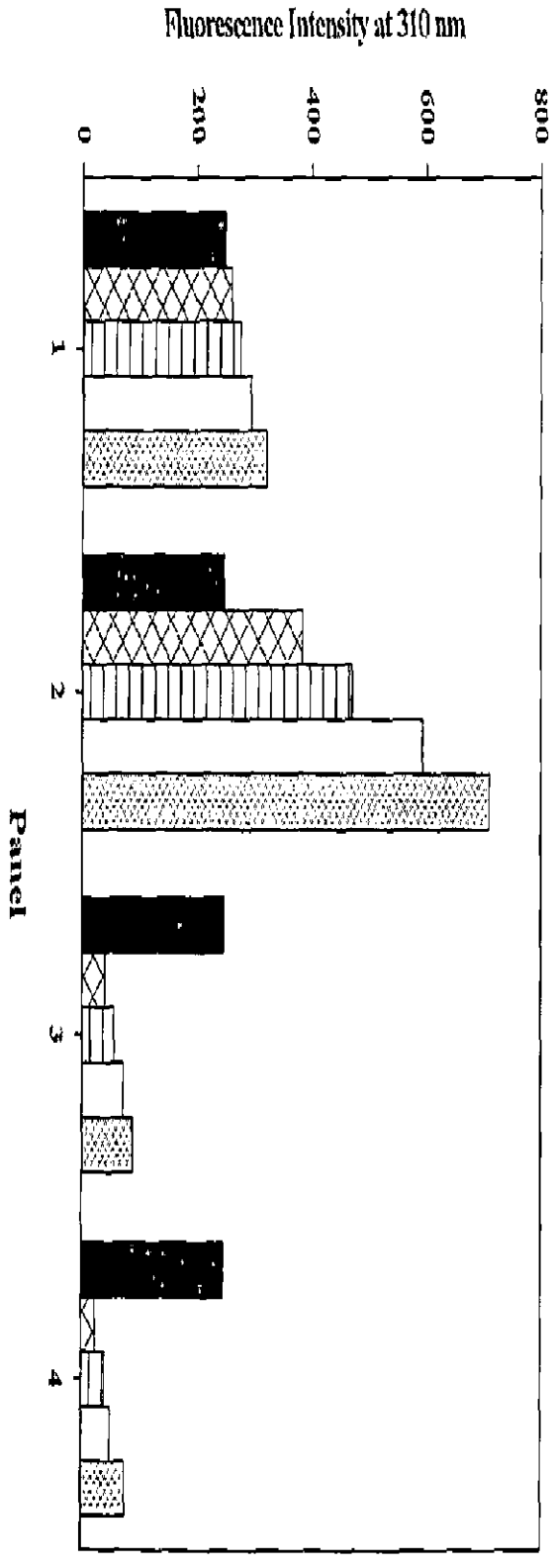

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Absorbance at 490 nm
1
2
3
4
Panel

Fig. 42. Effect of EA on the intrinsic fluorescence changes induced in SOD due to glycation. Fluorescence intensity at the excitation/emission wavelengths of 280/310 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM EA. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).


Fluorescence Intensity at 310 nm
0
200
400
600
800
Panel
1
2
3
4

4.5.6. AGEs specific fluorescence studies

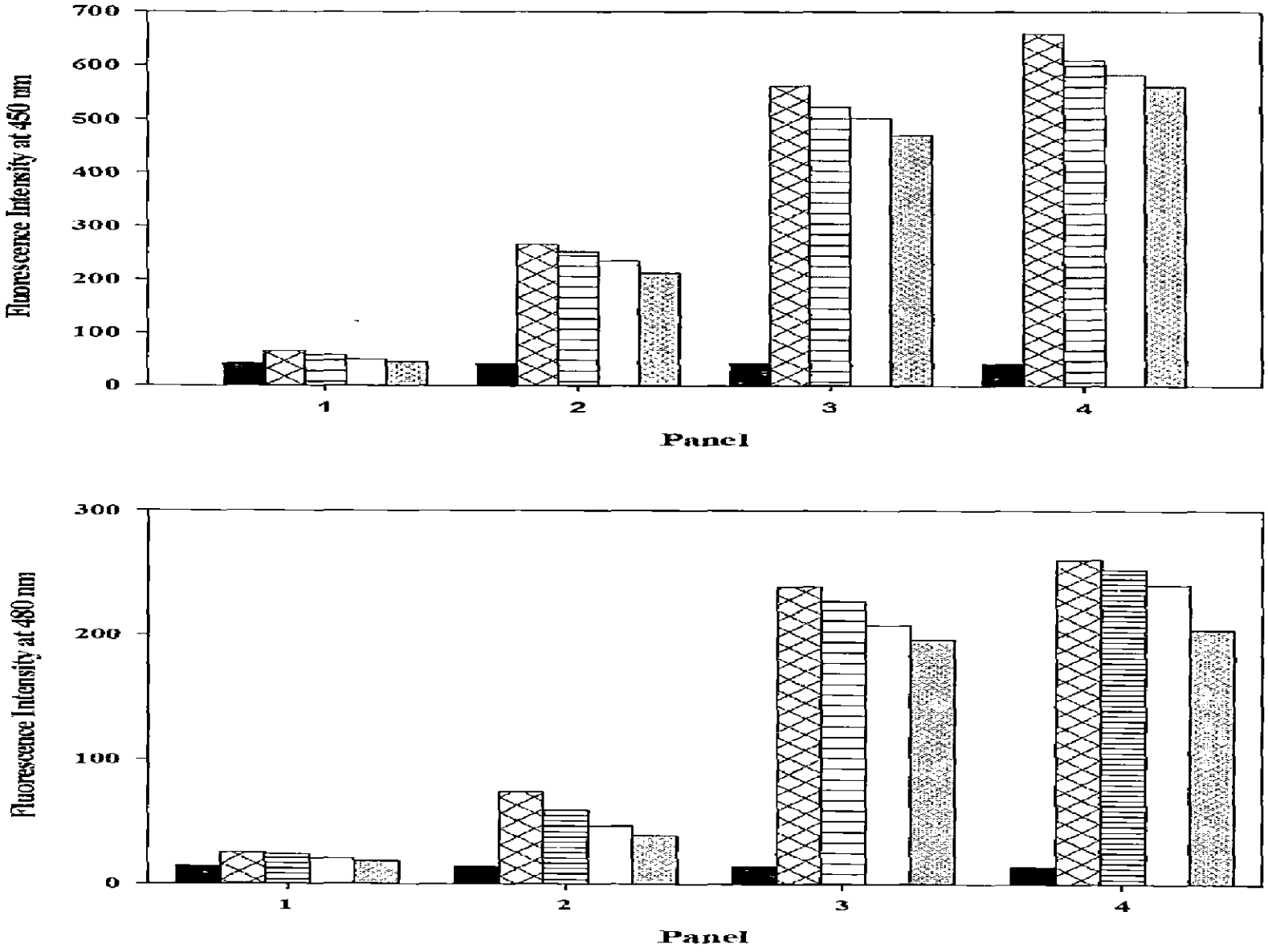
When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of EA (Fig. 43, Panel 2, 3 and 4, respectively), a progressive decrease in AGEs specific fluorescence at 450 nm with increasing EA concentration was observed in all the three cases. The control exhibited insignificant decrease in fluorescence at 450 nm (Fig. 43, Panel 1). Therefore, EA protected the enzyme to some extent against formation of AGEs induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of EA.

4.5.7. ThT fluorescence studies

Glycation of SOD by glucose, MG and both glucose and MG results in ThT fluorescence enhancement at 480 nm (formation of fibrils). When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of EA (Fig. 44, Panel 2, 3 and 4, respectively), a progressive decrease in ThT fluorescence with increasing EA was observed in all the three cases. The control exhibited insignificant decrease in ThT fluorescence at 480 nm (Fig. 44, Panel 1). Therefore, EA protected the enzyme to some extent against formation of fibrils induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of EA.

Fig. 43. Effect of EA on the fluorescent AGEs formed of SOD due to glycation. AGEs specific fluorescence intensity at the excitation/emission wavelengths of 350/450 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM EA. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).

Fig. 44. Effect of EA on the fibrils formed in SOD due to glycation. ThT fluorescence intensity at the excitation/emission wavelengths of 440/480 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM EA. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).


Fluorescence Intensity at 450 nm
700
600
500
400
300
200
100
0
1
2
3
4
Panel
Fluorescence Intensity at 480 nm
300
200
100
0
1
2
3
4
Panel

4.6. Protective effect of alliin on the glycation of SOD with glucose or MG

4.6.1. Activity studies

The effect of alliin on the activity of SOD glycated by 0.5 M glucose or 10 mM MG was studied. Fig. 45 shows the percentage remaining activity of SOD incubated for ten days at 37°C alone, and that incubated in the presence of glucose, MG or a combination of both and with increasing concentration of alliin. SOD incubated for ten days alone with alliin showed a slight increase in activity with increasing alliin concentration (Fig. 45, Panel 1). The activity increased by 5.1% when the enzyme was incubated with 50 μM alliin respectively as compared to the control (the sample that had no alliin). This slight increase in activity of SOD is believed to be due the antioxidant property of alliin. SOD incubated with glucose, MG or a combination of both, and alliin showed a greater increase in activity as compared to the enzyme that was not incubated with glucose or MG. The activity increased by 15.1, 14.3 and 10.6% as compared to the control when the enzyme was incubated with glucose (Fig. 45, Panel 2), MG (Fig. 45, Panel 3) or a combination of both glucose and MG (Fig. 45, Panel 4), respectively, and 50 μM alliin. This observed further increase in activity is believed to be due to the antiglycating activity of alliin. The data clearly indicates that the increase in activity was more in the case of glucose than for MG or both MG and glucose. Therefore, alliin is a more effective antiglycating agent for sugars/compounds that are milder glycating agents.

4.6.2. SDS-PAGE

The protective effect of alliin on SOD fragmentation/cross-linking induced by glycation is seen in Fig. 46. SDS-PAGE of SOD incubated for ten days in the absence of glucose or MG showed same staining intensity with increasing alliin concentration (Fig. 46A). However, the enzyme incubated for ten days with glucose showed a more increase in staining intensity with increasing alliin concentration (Fig. 46B). SOD incubated for ten days with MG (Fig. 46C) or a combination of both glucose and MG (Fig. 46D) exhibited a decrease in the bands corresponding to the cross-linked aggregates with increasing alliin concentration. Infact in the case of MG alone, there was a slight increase in the band corresponding to the native enzyme with increasing alliin concentration. Therefore, it is evident from SDS-PAGE analysis that alliin protected SOD against fragmentation/cross-linking induced by glycation.

Fig. 45. Effect of alliin on the activity of SOD incubated with glucose, MG or both. SOD (0.2 mg/ml) was incubated alone (Panel 1), with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM alliin. Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■). The enzyme activity was then determined under standard assay conditions. Each value represents the average for three independent experiments performed in duplicates.

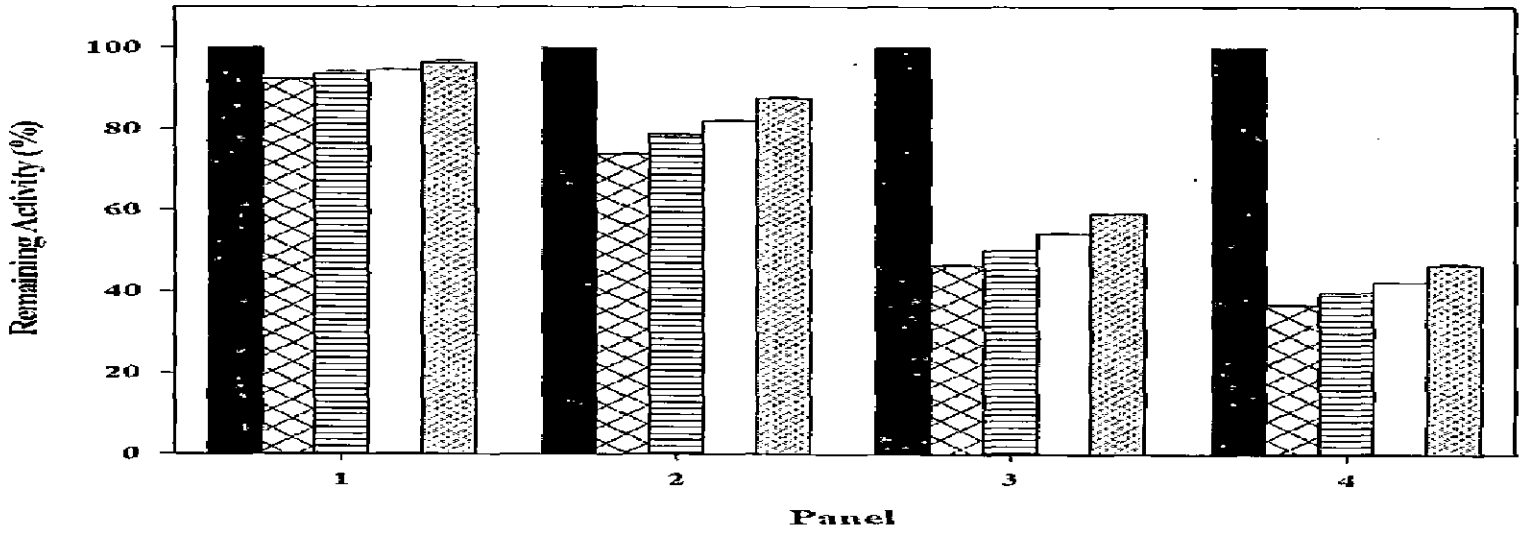
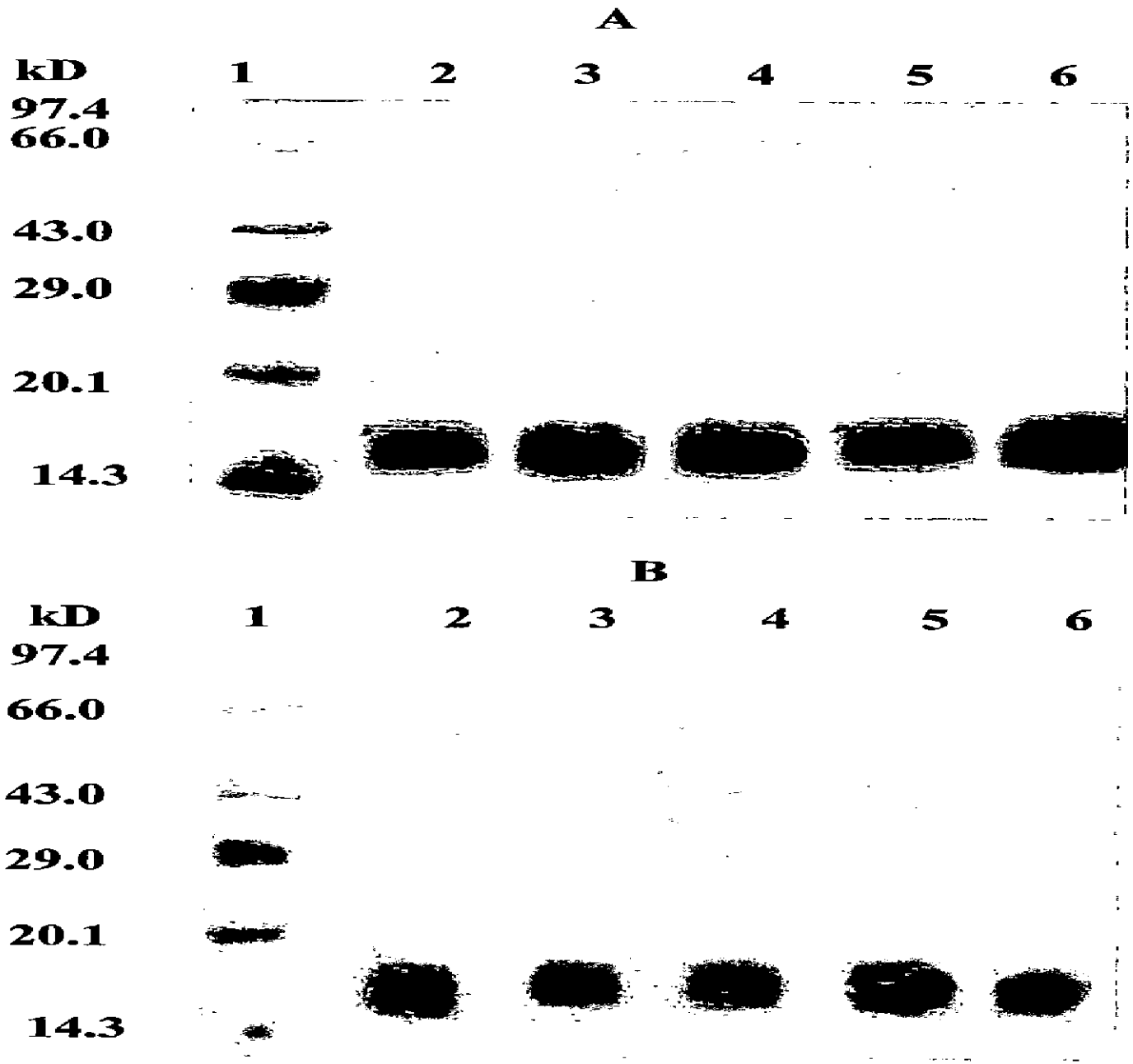
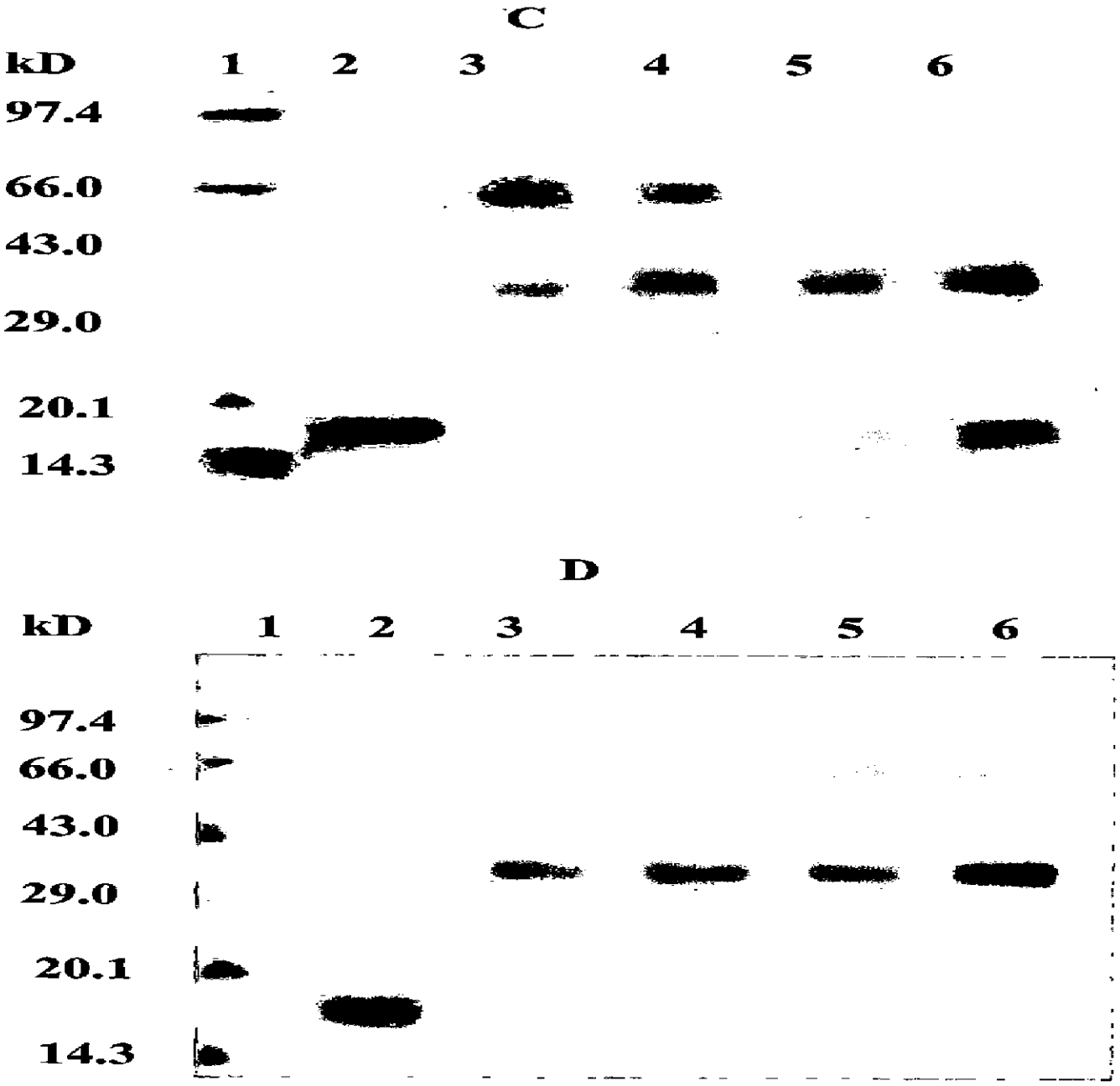

Remaining Activity (%)
100
80
60
40
20
0
1
2
3
4
Panel

Fig. 46. SDS-PAGE of SOD incubated alone (A), with 0.5 M glucose (B), with 10 mM MG (C) or a combination of 0.5 M glucose and 10 mM MG (D) and with varying concentration of alliin for 10 days at 37^{0}C. Lane 1 shows molecular weight markers (Genei); Lane 2 shows SOD (10 μg) that has not been incubated with glucose, MG or alliin. Lanes 3, 4, 5 and 6 show SOD (10 μg) incubated for 10 days alone or with glucose, MG or a combination of glucose and MG and with 0, 10, 20 and 50 μM alliin, respectively.


A
kD
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3
B
kD
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3


C
kD
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3
D
kD
1
2
3
4
5
6
97.4
66.0
43.0
29.0
20.1
14.3

4.6.3. ELISA

When SOD is incubated for 10 days at 37°C alone or with glucose, MG or both glucose and MG and increasing concentration of alliin, a progressive increase in absorbance at 490 nm (cross-reactivity with anti-SOD antibodies) with increasing alliin concentration (Fig. 47) was observed in all the four cases. Therefore, this experiment shows that alliin protected the enzyme to some extent against the structural/chemical changes induced by incubation at 37°C and glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of alliin.

4.6.4. UV absorption studies

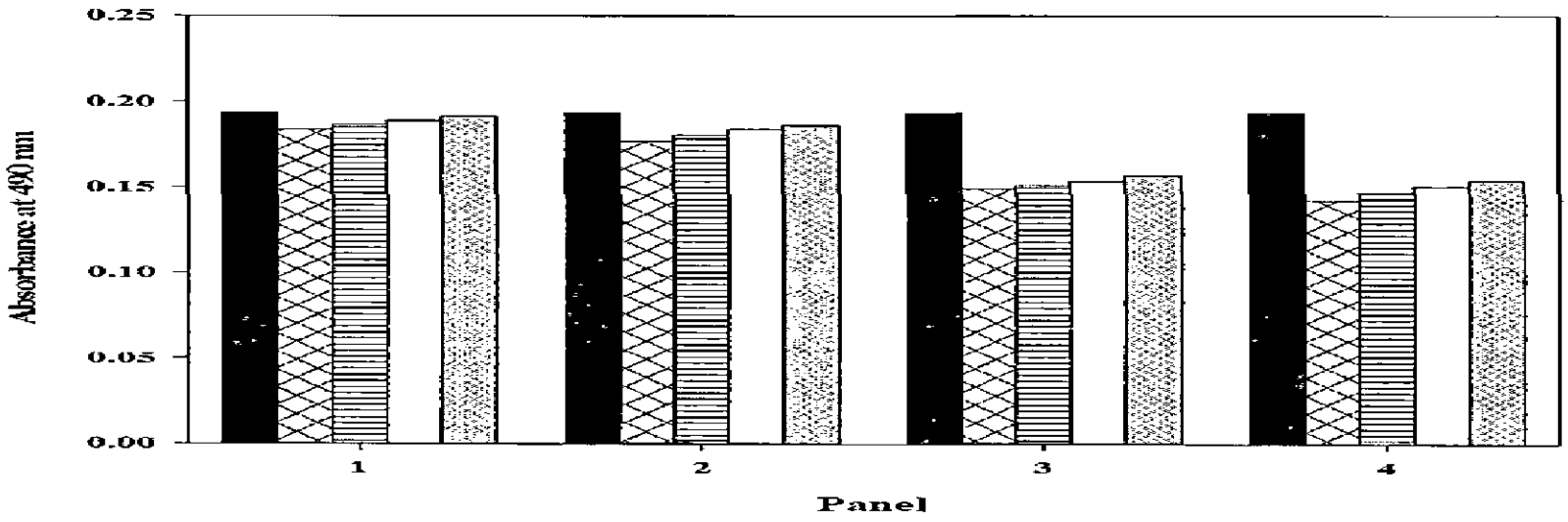
When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of alliin, a progressive decrease in absorbance at 280 nm with increasing alliin (Fig. 48, Panel 2, 3 and 4, respectively) was observed in all the three cases. SOD incubated alone for 10 days with increasing alliin concentration (control) exhibited very slight decrease in absorbance at 280 nm (Fig. 48, Panel 1). Therefore, alliin protected the enzyme to some extent against the structural changes induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of alliin. However, the enzyme was still far from the structure of the native enzyme even at 50 μM concentration of alliin, respectively, in all the three cases.

4.6.5. Intrinsic fluorescence studies

When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of alliin, a progressive increase in fluorescence at 310 nm with increasing alliin concentration was observed in all the three cases (Fig. 49, panel 2, 3, and 4, respectively). The control exhibited insignificant increase in fluorescence at 310 nm (Fig. 49, panel 1). Therefore, again this experiment shows that alliin protected the enzyme to some extent against the structural changes induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of alliin. The earlier results where fluorescence enhancement at 310 nm instead of quenching for SOD incubated for 10 days at 37°C with glucose and 1% DMSO were explained on the assumption that in the sample of

Fig. 47. Effect of alliin on the cross-reactivity (ELISA) of anti-SOD antibodies with SOD incubated with glucose, MG or both. SOD (0.2 mg/ml) was incubated alone (Panel 1), with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM alliin. Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■). Each value represents the average for two independent experiments performed in triplicates.

Fig. 48. Effect of alliin on the absorption changes induced in SOD due to glycation. Absorbance at 280 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM alliin. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).


Absorbance at 490 nm
0.25
0.20
0.15
0.10
0.05
0.00
1
2
3
4
Panel

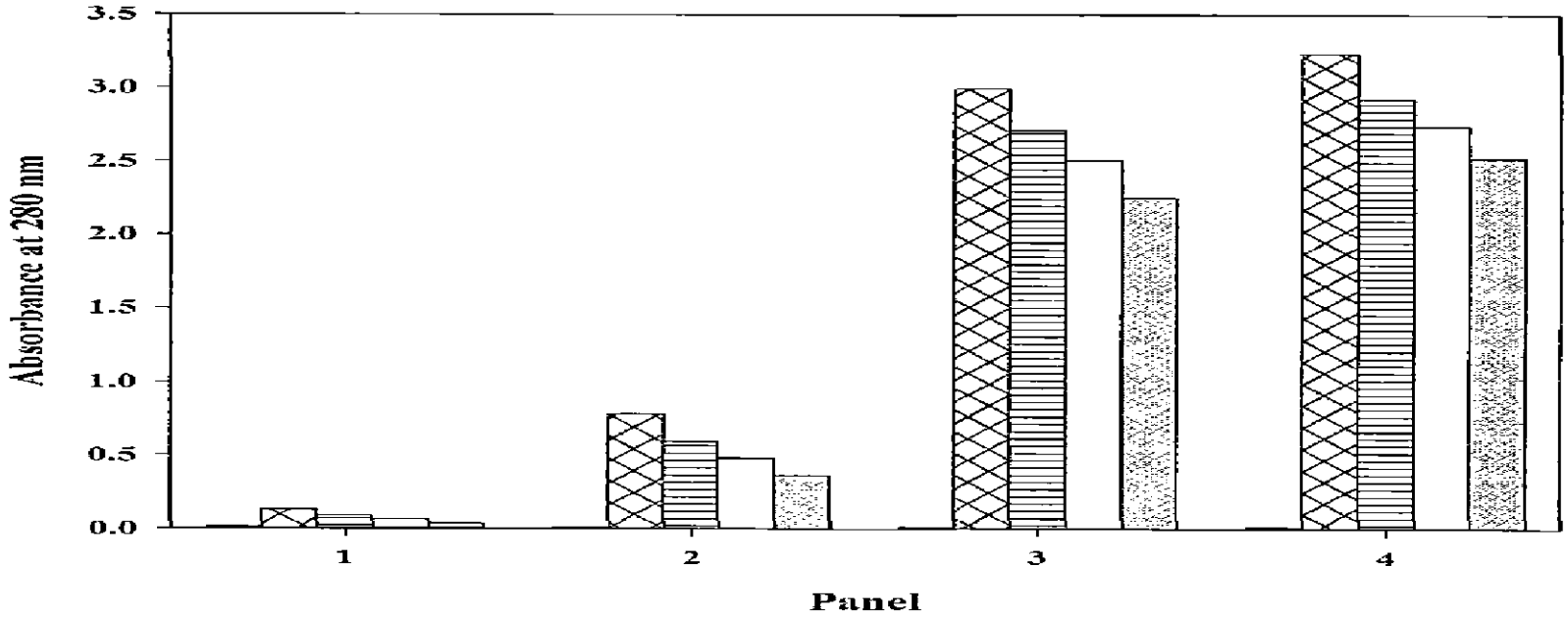
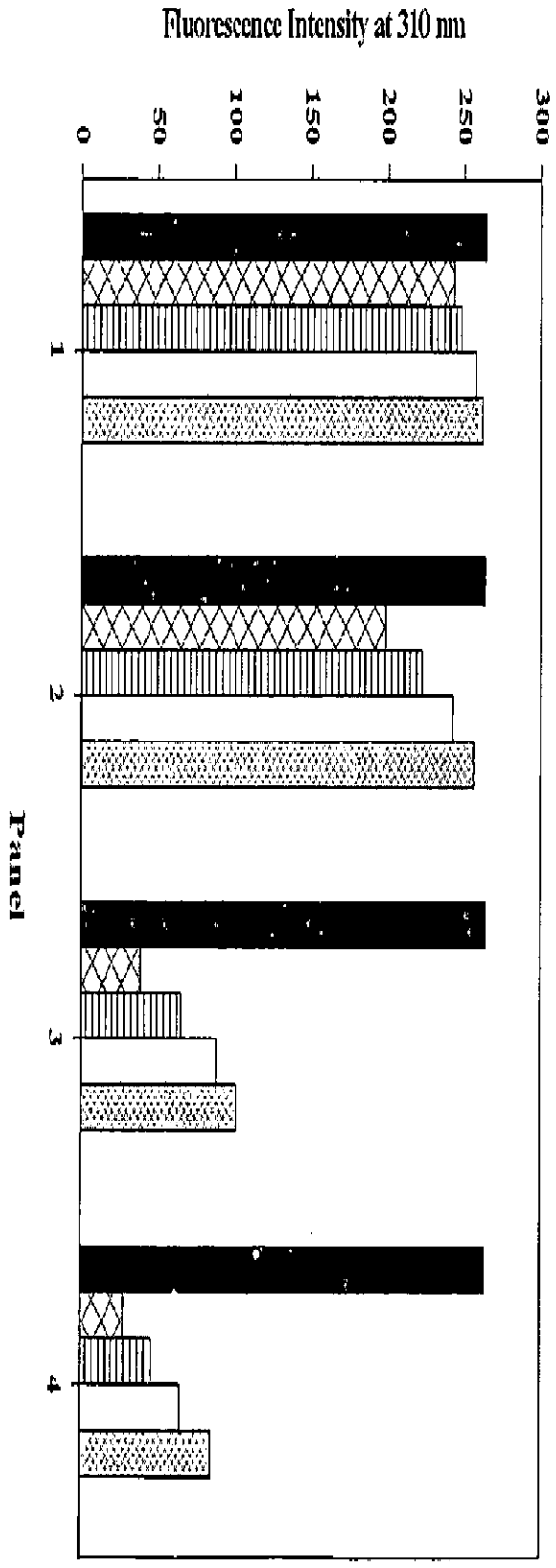

Absorbance at 280 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
Panel

Fig. 49. Effect of alliin on the intrinsic fluorescence changes induced in SOD due to glycation. Fluorescence intensity at the excitation/emission wavelengths of 280/310 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM alliin. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).


Fluorescence Intensity at 310 nm
0
50
100
150
200
250
300
Panel
1
2
3
4

SOD glycated by glucose in the presence of DMSO, the environment around the aromatic residues of the protein is somewhat perturbed which affects the fluorescence. However, in the present experiment, alliin was dissolved in buffer and no DMSO was present in the sample, therefore as expected quenching was observed. Therefore, this proves our earlier assumption.

4.6.6. AGEs specific fluorescence

When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of alliin (Fig. 50, Panel 2, 3 and 4, respectively), a progressive decrease in AGEs specific fluorescence at 450 nm with increasing alliin concentration was observed in all the three cases. The control exhibited insignificant decrease in fluorescence at 450 nm (Fig. 50, Panel 1). Therefore, alliin protected the enzyme to some extent against formation of AGEs induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of alliin.

4.6.7. ThT fluorescence studies

When SOD is incubated for 10 days at 37°C with glucose, MG or both glucose and MG and increasing concentration of alliin (Fig. 51, Panel 2, 3 and 4, respectively), a progressive decrease in ThT fluorescence with increasing alliin was observed in all the three cases. The control exhibited insignificant decrease in ThT fluorescence at 480 nm (Fig. 51, Panel 1). Therefore, alliin protected the enzyme to some extent against formation of fibrils induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of alliin.

4.6.8. CD studies

Far-UV CD studies which were not possible for TQ, *A. vera*, aloin and EA due to DMSO in the samples which interfered with the measurements in this wavelength range, were possible for alliin as in this case no DMSO was present in the samples. Glycation of SOD by glucose, MG and both glucose and MG results in decrease in the negative ellipticity (secondary structural changes). When SOD is incubated for 10 days at 37°C alone (control) or with glucose, MG or both glucose and MG and increasing concentration of alliin, a progressive increase in the negative ellipticity with increasing alliin concentration was observed in all the four cases (Fig. 52 Panel 1, 2, 3 and 4, respectively). Therefore, alliin protected the enzyme to some extent

Fig. 50. Effect of alliin on the fluorescent AGEs formed of SOD due to glycation. AGEs specific fluorescence intensity at the excitation/emission wavelengths of 350/450 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM alliin. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).

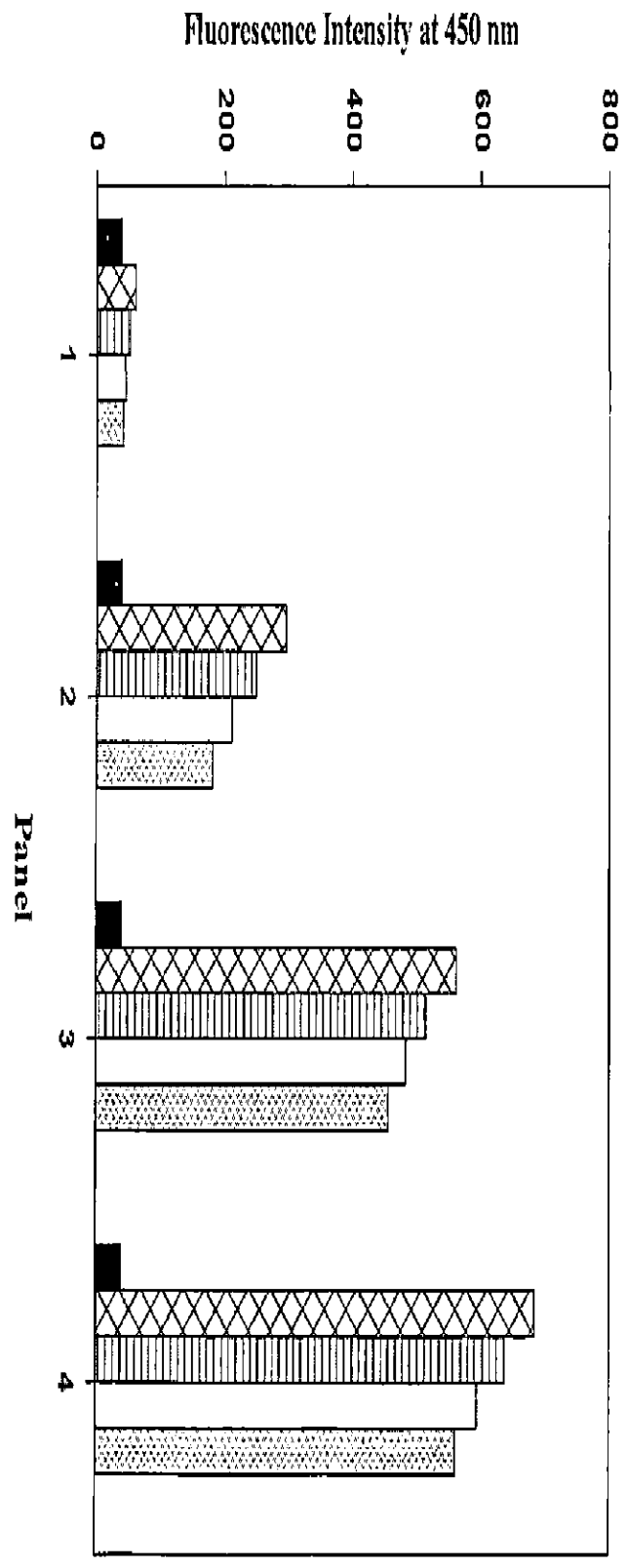

Fluorescence Intensity at 450 nm
0
200
400
600
800
Panel
1
2
3
4

Fig. 51. Effect of alliin on the fibrils formed in SOD due to glycation. ThT fluorescence intensity at the excitation/emission wavelengths of 440/480 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μM alliin. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).

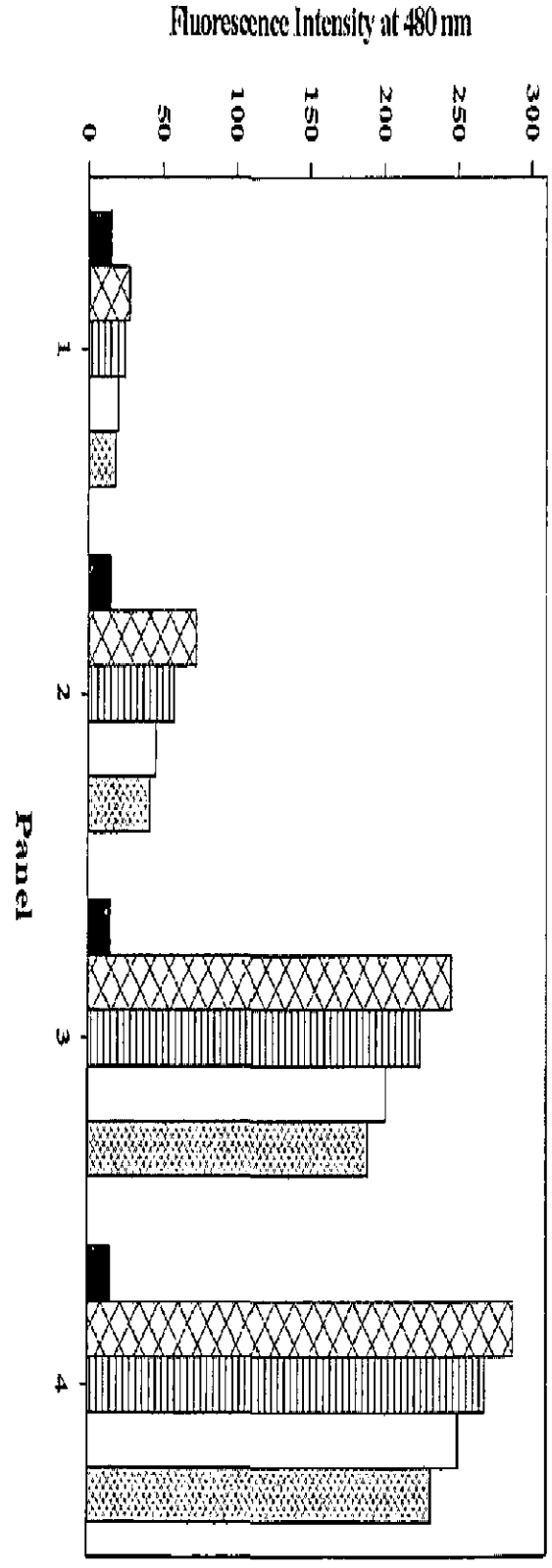
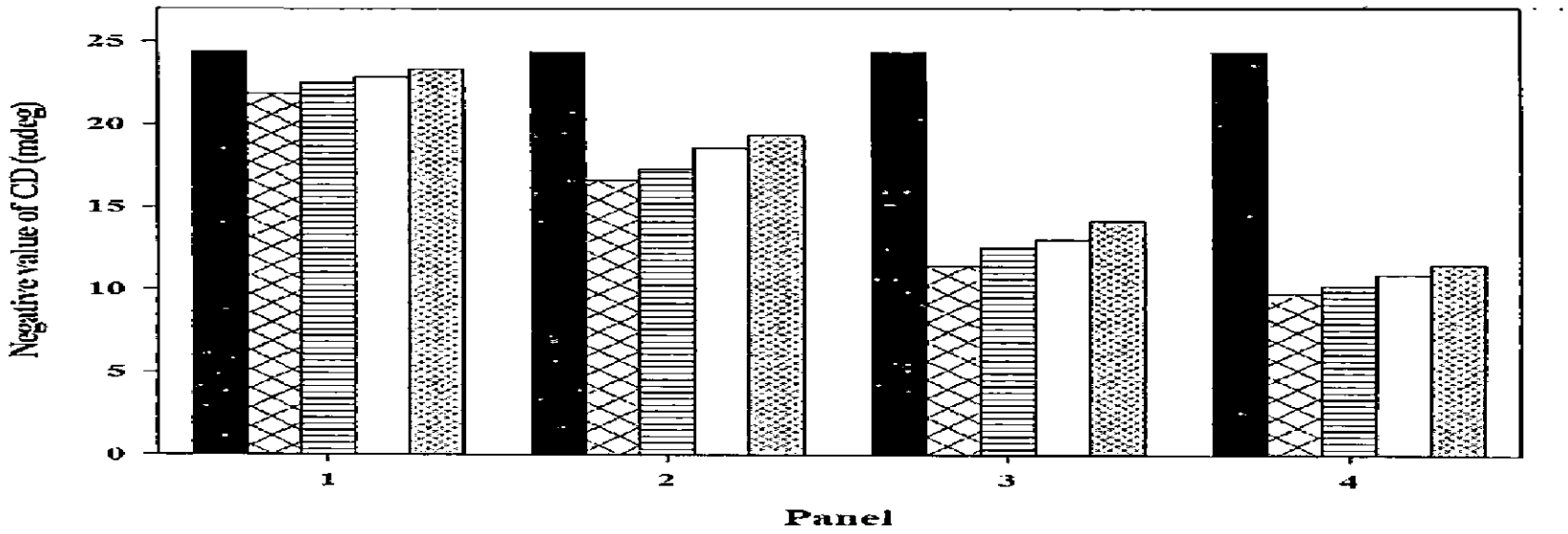

Fluorescence Intensity at 480 nm
0
50
100
150
200
250
300
Panel
1
2
3
4

Fig. 52. Effect of alliin on the far-UV CD changes induced in SOD due to glycation. CD at 208 nm for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (♯), 10 (≡), 20 (□) and 50 (▒) μg/ml of alliin. Each Panel also shows native SOD alone in buffer that was not incubated at 37°C for any time period (■).


Negative value of CD (mdeg)
25
20
15
10
5
0
1
2
3
4
Panel

against the secondary structural changes induced by incubation at 37°C and glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of alliin.

4.7. Activity studies on comparative antiglycating potential of TQ, aloin, EA, alliin, and quercetin

Quercetin has been reported to be a potent natural inhibitor of glycation (Sero *et al.*, 2013). Therefore, we compared the antiglycating potential of the natural compounds used in this study, TQ, aloin, EA and alliin with that of quercetin by activity measurements. The activity increased by 17.3, 18.2 and 11.0% as compared to the control when the enzyme was incubated with glucose, MG or a combination of both glucose and MG, respectively, and 50 μM quercetin (Fig. 53). The comparative antiglycating potential of TQ, aloin, EA, alliin and quercetin by activity measurements is shown in Table 1. The compound showing the best antiglycating potential is quercetin and the one showing the least is TQ. The order of decrease of antiglycating potential is: Quercetin > EA/alliin > aloin > TQ. However, examining the values, the antiglycating potential of EA and alliin appears to be comparable with that of quercetin.

4.8. Conclusions

The study shows that the natural products used in this study (TQ, *A. vera*, aloin, EA and alliin), which have previously been reported to have several beneficial pharmacological activities, also have antiglycating activity. The antiglycating activity appears to be better for mild glycating agents. These natural products also protect against potent glycating agents such as MG. These natural products have also been reported to have antidiabetic effects. This taken together with their antiglycating effect as observed in this study makes them effective antidiabetic products which can be used in treating diabetes and its complications.

Fig. 53. Effect of quercetin on the activity of SOD incubated with glucose, MG or combination of both glucose and MG. SOD (0.2 mg/ml) was incubated alone (Panel 1), with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37°C in the presence of 0 (#), 10 (≡), 20 (□) and 50 μM (▒) quercetin. Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37°C for any time period (■). The enzyme activity was then determined under standard assay conditions. Each value represents the average for three independent experiments performed in duplicates.

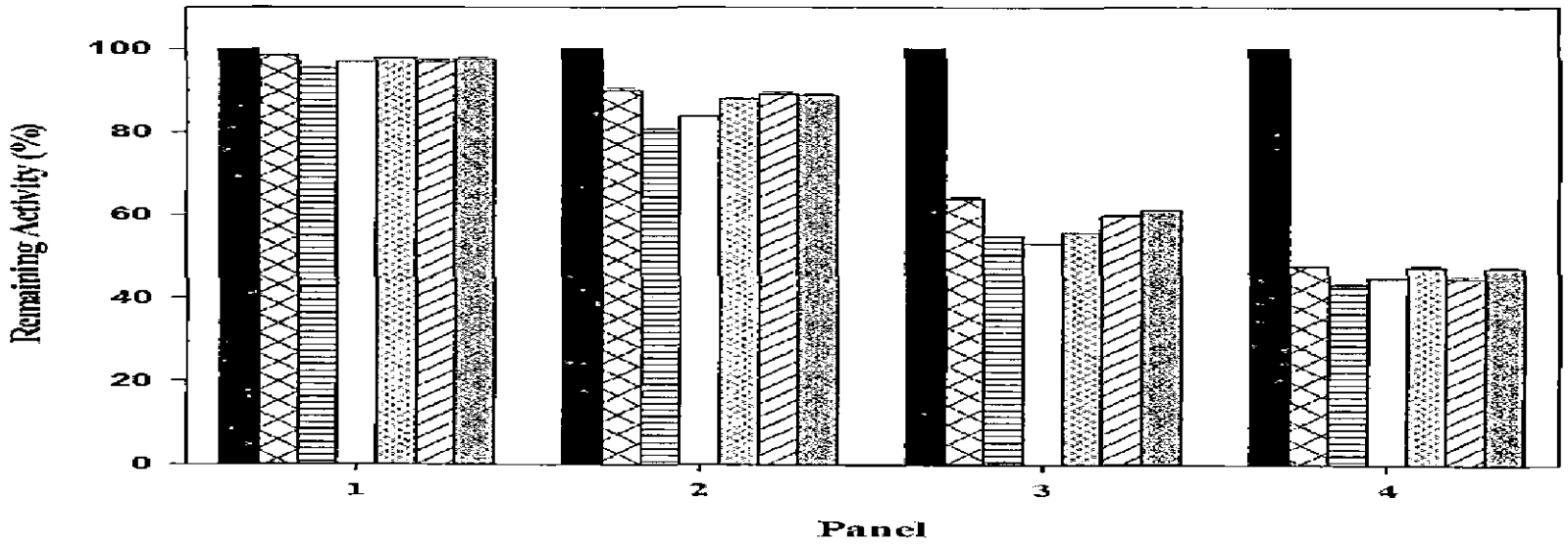

Remaining Activity (%)
100
80
60
40
20
0
1
2
3
4
Panel

Table 1: Comparative antiglycating potential of natural compounds. The activity increase as compared to the control when SOD was incubated with glucose, MG or combination of both glucose and MG and 50 μM natural compound is shown.

Natural compound	Activity increase when SOD was incubated with:		
	Glucose	**MG**	**Glucose + MG**
TQ	9.6	7.7	6.2
Aloin	14.6	10.1	9.8
EA	15.9	13.7	10.0
Alliin	15.1	14.3	10.6
Quercetin	17.3	18.2	11.0

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Appendices

ST OF PAPERS/ABSTRACTS

1. Protective effect of thymoquinone on glucose or methylglyoxal-induced glycation of superoxide dismutase. Masood A. Khan[1], Shehwaz Anwar[1], Ahmad N. Alijarbou, Mohammad Al-Orainy, Yosef H Aldebasi, Sehbanul Islam, Hina Younus. *International Journal of Biological Macromolecules.* 65 (2014) 16-20.

2. A structural study on the protection of glycation of superoxide dismutase by thymoquinone. Shehwaz Anwar, Masood Alam Khan, Ayesha Sadaf, HinaYounus.

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3. Abstract in national symposium (2012) in Interdisciplinary Biotechnology Unit, A.M.U., Aligarh. Topic: Glycation of superoxide dismutase by glucose and methylglyoxal.

4. Abstract in 3rd Annual Meeting (2013) Indian Academy of Biomedical sciences & Symposium on Modern Trends in Human Diseases. Topic: Effect of black seed component thymoquinone on glucose or methylglucose-induced glycation of superoxide dismutase.

5. Abstract of oral presentation in National Seminar (2014) in Department of Biosciences, Jamia Milia Islamia, New Delhi. Topic: Protective effect of Aloe Vera gel extract on the glycation of superoxide dismutase.

6. Abstract of Oral presentation in National Symposium cum Bioinformatics workshop in Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh. Topic: Protective effect of Aloe vera leaf gel component aloin on the glycation of superoxide dismutase.

7. Effect of divalent cations on the activity and conformation of yeast alcohol dehydrogenase. Rizwanul Haque, **Shehwaz Anwar**, Md. Fazle Alam, Hina Younus. Journal of Protein and Proteomics. 3(2012) 113-118.

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Protective effect of thymoquinone on glucose or methylglyoxal-induced glycation of superoxide dismutase

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ABSTRACT

Glycation plays an important role in various oxidative stress related diseases. Superoxide dismutase (SOD) constitutes an essential defense against oxidative stress. The damage caused by oxidative stress is exacerbated if the antioxidant enzymes themselves are inactivated by glycation. Thymoquinone (TQ) has been reported to have various pharmacological activities. Therefore, the glycation of SOD by glucose or methylglyoxal (MG) and its protection by TQ has been investigated. Incubation of SOD with glucose, MG or both at 37 °C resulted in a progressive decrease in the activity of the enzyme, and a parallel decrease in the amount of protein on SDS-PAGE gels for glucose incubated SOD and formation of high molecular weight aggregates for MG or both glucose and MG incubated enzyme. TQ offered protection against glucose or MG induced loss in SOD activity and fragmentation/cross-linking. The antiglycating activity of TQ appears to be better for mild glycating agents. It is also effective in protecting against strong glycating agents, more when the exposure time to the glycating agent is short. TQ has also earlier been reported to have anti-diabetic effects, and this along with the observed antiglycating effect makes it an effective compound against diabetes and its complications.

1. Introduction

Diabetes has become the most common metabolic disease worldwide. Hyperglycemia has a key role in the diseases associated with diabetic complications. Non-enzymatic glycation comprises a complex series of reactions between reducing sugars and amino groups of proteins, lipids and nucleic acids. During glycation, the carbonyl groups of sugars react slowly with the free amino groups yielding Schiff base. The Schiff bases undergo Amadori rearrangement, and through a series of further rearrangements, cyclizations, dehydrations, etc. form a variety of diverse compounds, collectively described as advanced glycation end products (AGEs) [1,2]. AGE formation is accompanied by the formation, among others of a number of reactive oxygen species, α-oxoaldehydes including MG, that further react and damage the proteins and other important biological molecules. MG reacts irreversibly with amino groups in proteins, forming AGEs [3]. Glycation of proteins play an important role in the development of physiological and pathophysiological processes, such as aging, diabetes, atherosclerosis, neurodegenerative diseases, vascular diseases and chronic renal failure [4].

SODs are a family of metalloenzymes that catalyze the dismutation of superoxide radicals (O_2^-) into molecular oxygen and H_2O_2 mediated by alternate oxidation-reduction of metal ions present at their active site [5,6]. Among the enzymes inactivated by glycation, SOD constitutes the first, and possibly, the most important line of antioxidant defense, enabling cells to cope with lethal oxidative environments. Exposure of SOD to glucose results in its deactivation following site-specific and random fragmentation [7]. The damage caused by oxidative stress is expected to be exacerbated if the antioxidant enzymes themselves are inactivated by glycation. Elevated levels of MG have been reported to adversely affect SODs particularly against superoxide radicals. Exposure of SOD to MG has been shown to cause its covalent cross-linking associated with loss of enzymatic activity [8].

Current anti-diabetic therapy is based on synthetic drugs that very often have side effects [9]. Alternative medicines and natural

Abbreviations: SOD, superoxide dismutase; TQ, thymoquinone; AGE, sadvanced glycation end products; MG, methylglyoxal; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NBT, nitro blue tetrazolium; PMS, phenazine methosulfate.

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therapies have stimulated new interest of research to find for more efficacious agents with lesser side effects. Naturally occurring phytochemicals with anti-diabetic activities are relatively nontoxic, inexpensive and available in an ingestable form. A large number of plants and natural biomolecules have been discussed in literature for their anti-diabetic effects [10–12]. The mechanism is most often not completely understood. A large number of hypoglycaemic compounds have anti-oxidant properties. Throughout history black seeds, *Nigella sativa* seeds, have been one of the most revered medicinal seeds in history. TQ, an active principle component of the volatile oil of black cumin seeds, possess anti-diabetic [13], anti-oxidant [14], hepatoprotective [15], neuroprotective [16], nephroprotective [17], anti-tumor [18] and anti-mutagenic [19] pharmacological activities. The aim of this study was to determine the anti-glycating effect of TQ. The glycation of SOD by glucose or MG and its protection by TQ has been studied by activity measurements and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2. Materials and methods

2.1. Materials

Bovine erythrocyte Cu,Zn-SOD (EC 1.15.1.1), MG (40% aqueous solution), nitro blue tetrazolium (NBT), NADH, phenazine methosulfate (PMS), bovine serum albumin (BSA), bicinchonic acid (BCA) and *N, N, N', N'*-tetramethylenediamine (TEMED) were purchased from Sigma, USA. Glucose, sodium dodecyl sulphate (SDS) was from Qualigens, India. TQ was a product of Aldrich. Acylamide/bisacrylamide and ammonium persulphate were obtained from SRL, India. Standard molecular weight protein markers (Broad range) were from Genei, India. All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Measurement of SOD activity

The activity of SOD was determined spectrophotometrically by employing PMS–NADH–NBT system [20]. The reaction mixture consisted of 20 mM sodium phosphate buffer (pH 8.2), PMS (1.9 μM), NBT (184 μM) and NADH (205 μM). For assaying, SOD enzyme was pipette into a cuvette at room temperature (25 °C) containing freshly prepared NBT and NADH. The reaction was initiated with the addition of freshly prepared PMS and the absorbance at 560 nm was continuously monitored as an index of NBT reduction using a single beam Shimadzu spectrophotometer. Reagent control lacking the enzyme was taken.

2.2.2. SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970) [21] using 15% separating and 5% stacking gels. Protein bands were visualized by overnight staining with 0.1% (w/v) Coomassie Brilliant Blue R250. The gels were destained using 40% (v/v) methanol/10% (v/v) acetic acid.

2.2.3. In vitro glycation of SOD by glucose, MG or a combination of both

SOD was dissolved in 20 mM sodium phosphate buffer, pH 7.4 to make a stock of 1 mg/ml. The protein concentration was determined using the BCA method using BSA as standard [22]. Stock was then stored at −20 °C for future use. In order to induce glycation, SOD (0.2 mg/ml) was incubated along with 0.5 M glucose, 10 mM MG or a combination of both 0.5 M glucose and 10 mM MG in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl. Incubations were performed in autoclaved tubes in order to maintain sterile conditions during the prolonged incubations. No bacterial growth was detected during the periods of incubation. The incubations were carried out in a shaking water bath at 37 °C for 1 h, 1 day, 5 days and 10 days, followed by assay of enzyme activity. The existence of free glucose or MG in the SOD protein solutions after incubation was found to have no effect on the enzyme activity at room temperature. Therefore, these solutions were not dialyzed after incubation. The native SOD incubated with sodium phosphate buffer (20 mM, pH 7.4 containing 0.15 M NaCl) alone served as the control. Glycation induced fragmentation and/or cross linking of SOD was assessed by SDS-PAGE.

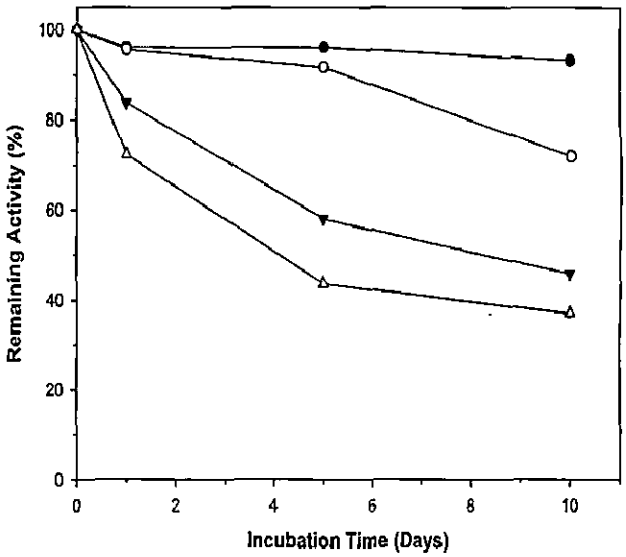

Fig. 1. Effect of glucose, MG or combination of both glucose and MG on the activity of SOD. SOD (0.2 mg/ml) was incubated with 0.5 M glucose (○), 10 mM MG (▼) or a combination of 0.5 M glucose and 10 mM MG (△) for 10 days under sterile conditions at 37 °C. Small aliquots were removed at appropriate intervals and enzyme activity was measured under standard assay conditions. SOD in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl incubated for 10 days under similar conditions served as the control (●). Each value represents the average for three independent experiments performed in duplicates.

2.2.4. Effect of TQ on the glycation of SOD by glucose, MG or a combination of both

SOD was incubated with 0.5 M glucose or 10 mM MG or a combination of both 0.5 M glucose and 10 mM MG for 10 days at 37 °C in the absence or presence of 10, 20 and 50 μM TQ. In a separate experiment, SOD was incubated with 10 mM MG for only 1 h at 37 °C in the absence or presence of 10, 20 and 50 μM TQ. The enzyme activity was then determined using the above standard assay. Glucose or MG or a combination of both induced fragmentation and/or cross linking of SOD in the absence/presence of TQ was assessed by SDS-PAGE.

3. Results and discussion

3.1. In vitro glycation of SOD by glucose, MG or both

Prolonged incubation of enzymes with sugars has been shown to result in their glycation and hence inactivation [23,24]. MG is a highly reactive α-oxoaldehyde that plays an important role in glycation reactions, formation of AGEs and other complications associated with hyperglycemia and related disorders [25]. SOD was incubated with high non-physiological concentration of glucose (0.5 M) or MG (10 mM) which may serve as an appropriate model for the long-term effects of glucose or MG on the enzyme [25,26]. The effect of 0.5 M glucose or 10 mM MG or a combination of 0.5 M glucose and 10 mM MG on the activity of SOD is shown in Fig. 1.

Incubation with glucose at 37 °C resulted in a decrease in the activity of SOD. At the end of ten days of incubation with glucose, the residual activity of the enzyme was 72%. MG which is more reactive than glucose in the maillard reaction inactivated SOD more rapidly. At the end of ten days of incubation with MG or a combination of both glucose and MG, the residual activity of SOD was 46% and 37%, respectively. SOD did not lose significant activity when incubated alone i.e in absence of glucose and MG. The residual activity in this case was 93% after ten days of incubation.

SDS-PAGE of SOD incubated at 37 °C for various days in the absence of glucose or MG exhibited very slight decrease in the intensity of the enzyme band (Fig. 2A), and this correlates with the fact that this enzyme preparation also retained 93% of activity after ten days of incubation. However, SOD incubated with glucose revealed a significant decrease in the staining intensity of the band corresponding to the enzyme (Fig. 2B). The band appeared more lighter with an increase in the duration of exposure to the sugar, presumably indicating its cross-linking and/or degradation into small peptides. Formation of high molecular weight cross-linked aggregates in the SOD exposed to MG or a combination of MG and glucose is evident from Fig. 2C and D, respectively. The high molecular weight bands started appearing within 1 h of incubation and their intensity increased with days of incubation. After ten days of incubation, no band corresponding to the free enzyme (uncross-linked) was visible in the gel. The effect of MG is thought to be due to its ability to form stable heterocyclic compounds that cross-link protein. The changes in the enzyme preparation exposed to both glucose and MG were slightly faster that exposed to only to MG. However, the effect of MG dominates over the effect of glucose, since MG is much more reactive and potent glycating agent than glucose.

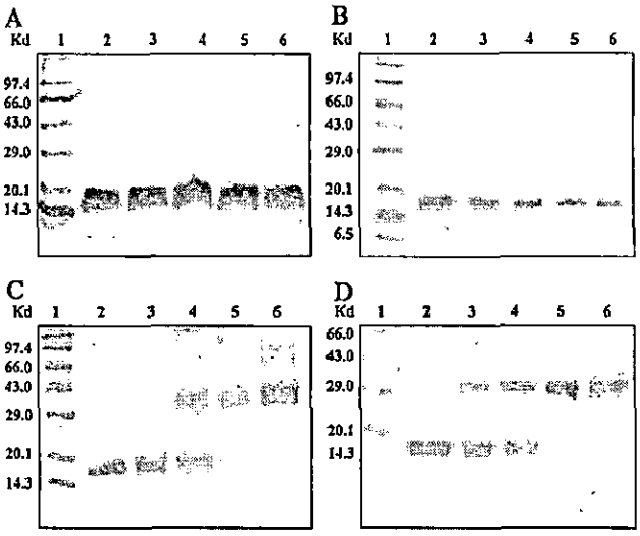

Fig. 2. SDS-PAGE of SOD incubated alone (A), with 0.5 M glucose (B), with 10 mM MG (C) or with a combination of 0.5 M glucose and 10 mM MG (D) for various time periods at 37 °C. Lane 1 shows molecular weight markers (Genei); Lanes 2, 3, 4, 5 and 6 show SOD (10 μg) incubated alone, with glucose, with MG or with a combination of glucose and MG for 0 h, 1 h, 1 day, 5 days and 10 days, respectively.

3.2. Protective effect of TQ on the glycation of SOD with glucose, MG or combination of both

The effect of TQ on the glycation of SOD by 0.5 M glucose or 10 mM MG was studied. Fig. 3 shows the remaining activity of SOD incubated for ten days at 37 °C alone, and that incubated in the

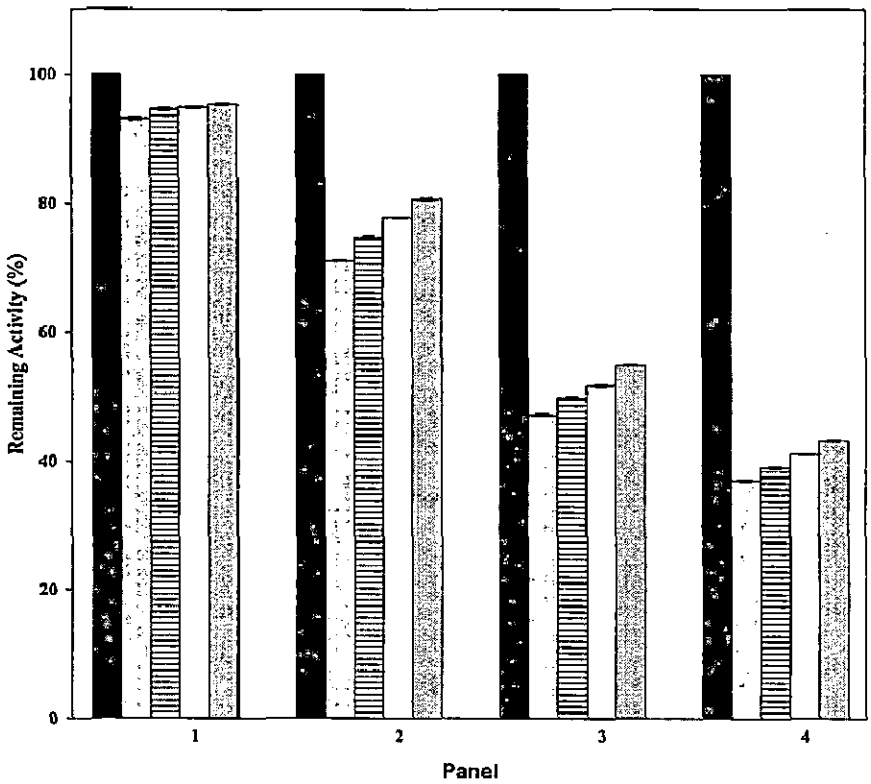

Fig. 3. Effect of TQ on the activity of SOD incubated with glucose, MG or combination of both glucose and MG. SOD (0.2 mg/ml) was incubated alone (Panel 1), with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37 °C in the presence of 0 (▨), 10 (□), 20 (▤) and 50 (#) μM concentration of TQ. Each Panel also shows native SOD (0.2 mg/ml) alone in buffer that was not incubated at 37 °C for any time period (■). The enzyme activity was then determined under standard assay conditions. Each value represents the average for three independent experiments performed in duplicates.

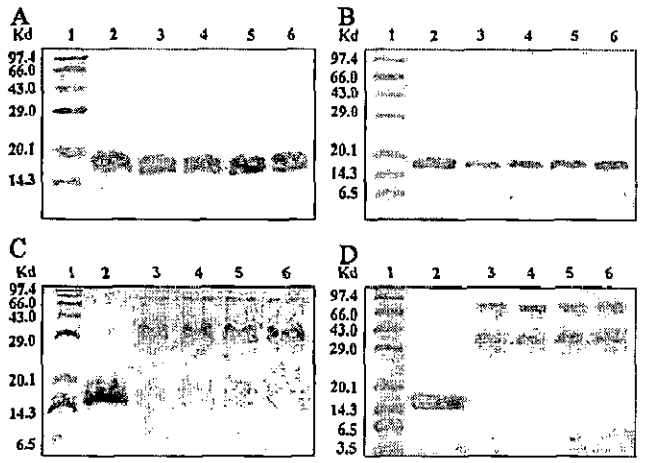

Fig. 4. SDS-PAGE of SOD incubated alone (A), with 0.5 M glucose (B), with 10 mM MG (C) or a combination of 0.5 M glucose and 10 mM MG (D) and with varying concentration of TQ for 10 days at 37 °C. Lane 1 shows molecular weight markers (Genei); Lane 2 shows SOD (10 μg) that has not been incubated with glucose, MG or TQ. Lanes 3, 4, 5 and 6 show SOD (10 μg) incubated for 10 days alone or with glucose, MG or a combination of glucose and MG and with 0, 10, 20 and 50 μM TQ, respectively.

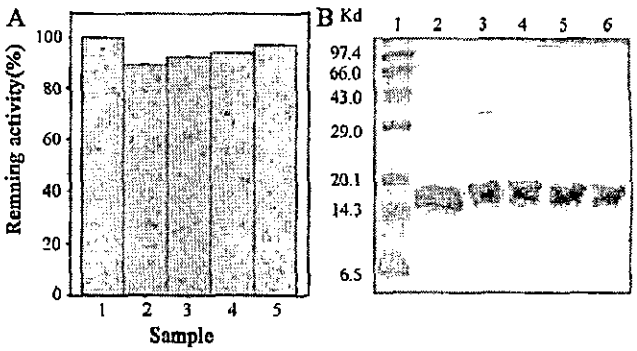

Fig. 5. Effect of TQ on the glycation of SOD incubated with MG for 1 h. A: SOD (0.2 mg/ml) was incubated with 10 mM MG for 1 h at 37 °C in the presence of 0, 10, 20 and 50 μM TQ (Sample 2, 3, 4 and 5, respectively). Sample 1 shows native SOD alone in buffer that was not incubated at 37 °C for any time period. The enzyme activity was then determined under standard assay conditions. Each value represents the average for three independent experiments performed in duplicates. B: SDS-PAGE of SOD (10 μg) incubated with 10 mM MG and TQ for 1 h at 37 °C. Lane 1 shows molecular weight markers (Genei); Lane 2 shows SOD that has not been incubated with MG or TQ. Lanes 3, 4, 5 and 6 show SOD incubated for 1 h with MG and with 0, 10, 20 and 50 μM TQ, respectively.

presence of glucose, MG or a combination of both and with increasing concentration of TQ. SOD incubated for ten days alone with TQ showed a slight increase in activity with increasing TQ concentration (Fig. 3 Panel 1). The activity increased by 2% when the enzyme was incubated with 50 μM TQ as compared to the control (the sample that had no TQ). This 2% increase in activity of SOD is believed to be due the antioxidant property of TQ. SOD incubated with glucose, MG or a combination of both and TQ showed a greater increase in activity as compared to the enzyme that was not incubated with glucose or MG. The activity increased by 10%, 8% and 6% as compared to the control when the enzyme was incubated with glucose (Fig. 3 Panel 2), MG (Fig. 3 Panel 3) or a combination of both glucose and MG (Fig. 3 Panel 4), respectively, and 50 μM TQ. This observed further increase in activity is believed to be due to the anti-glycating activity of TQ. The increase in activity was more in the case of glucose than for MG or both MG and glucose. Therefore, it appears that TQ is a more effective anti-glycating agent for sugars/compounds that are milder glycating agents.

The protective effect of TQ on SOD fragmentation/cross-linking induced by glycation is seen in Fig. 4. SDS-PAGE of SOD incubated for ten days in the absence of glucose or MG showed same staining intensity with increasing TQ concentration (Fig. 4A). However, the enzyme incubated for ten days with glucose showed a more increase in staining intensity with increasing TQ concentration (Fig. 4B). SOD incubated for ten days with MG (Fig. 4C) or a combination of both glucose and MG (Fig. 4D) exhibited a very slight decrease in the bands corresponding to the cross-linked aggregates with increasing TQ concentration. Infact in the case of MG alone, there was a slight increase in the band corresponding to the native enzyme with increasing TQ concentration. Therefore, it is evident from SDS-PAGE analysis that TQ slightly protected SOD against fragmentation/cross-linking induced by glycation. The protection appears to be in the following order for the enzyme incubated with glucose > MG > glucose + MG. This correlates with the activity observations.

Since SOD is glycated rapidly with MG, we studied the protective effect of TQ on the glycation of SOD incubated with MG only for 1 h. The activity increased by 8% as compared to the control when the enzyme was incubated with MG and 50 μM TQ for 1 h (Fig. 5A). The activity increase was also 8% when the enzyme was incubated with MG and 50 μM TQ for 10 days (Fig. 3 Panel 3). However, since the decrease in activity of SOD incubated with MG for 1 h was much lower (11%) than that incubated for 10 days (47%), this 8% increase in activity in the case of the former implies that TQ has a much more protective effect on the glycation of SOD by MG at short incubation time (1 h). SOD incubated for 1 h with MG shows a thin band corresponding to the cross-linked aggregates in SDS-PAGE, whose intensity was observed to decrease with increasing TQ concentration (Fig. 5B). At 50 μM TQ concentration, no cross-linked aggregates were observed. Both activity and SDS-PAGE analysis shows that TQ protects SOD against MG induced glycation to a much better extent at shorter glycation incubation time. Therefore, it implies that TQ also offers protection against strong glycating agents such as MG, more when the exposure time to the glycating agent is short.

3.3. Conclusions

This study shows that the active principle component of black cumin seed oil, TQ, which has previously reported to have several beneficial pharmacological activities, also has antig-lycating activity. The antig-lycating activity appears to be better for mild glycating agents. TQ also protects against potent glycating agents such as MG especially when the exposure time to the glycating agent is short. Therefore, black seed oil or TQ can be used for reducing diabetic complications many of which are due to protein glycation. TQ has also been reported to have anti-diabetic effects. Cumin seed extract inhibited the intestinal absorption of glucose [10], and increased insulinemia and HDL cholesterol [27]. TQ mediated a decrease in hepatic gluconeogenesis in diabetic hamsters [28]. This taken together with the antiglycating effect of TQ as observed in this study makes it an effective anti-diabetic compound which can be used in treating diabetes.

Acknowledgments

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A structural study on the protection of glycation of superoxide dismutase by thymoquinone

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ABSTRACT

Accumulation of advanced glycation end products (AGEs) in tissues and serum plays important roles in diabetes-associated complications. Therefore, the identification of antiglycating compounds is attracting considerable interest. In this study, the structural changes associated with the glycation of superoxide dismutase (SOD) and its protection by thymoquinone (TQ) have been investigated by biophysical techniques. Incubation of SOD with glucose, methylglyoxal (MG) or both at 37 °C resulted in progressive hyperchromicity at 280 nm, intrinsic fluorescence quenching at 310 nm, decrease in negative ellipticity at 208 nm, AGE-specific fluorescence enhancement in the wavelength range 400–480 nm and Thioflavin T (ThT) fluorescence enhancement at 480 nm (fibrillar state enhancement). Therefore, glycation by glucose or MG induced both tertiary and secondary structural changes in SOD and formation of AGEs and fibrils. The changes were more and faster with MG than with glucose since MG is a stronger glycating agent than glucose. TQ offered protection against glucose or MG-induced glycation of SOD as observed by a reduction in the structural changes, formation of AGEs and fibrils. Thus, TQ can be used for reducing diabetic complications many of which are due to protein glycation.

1. Introduction

Hyperglycaemia leading to the glycation of proteins, lipids and nucleic acids has an important role in the development of diseases, such as diabetes, atherosclerosis, neurodegeneration, chronic renal failure and vascular diseases [1]. During glycation, reducing sugars react with the amino groups of lysine side chains and the terminal amino groups of proteins to form unstable Schiff bases which rearrange to form more stable Amadori products (Fig. 1). These products further undergo a complex series of chemical reactions via dicarbonyl intermediates to a number of diverse compounds, collectively designated as advanced glycation end products (AGEs) [2,3] (Fig. 1). The post-Amadori products constitute highly reactive α-dicarbonyl intermediates including methylglyoxal (MG) that further damage the proteins and other biomolecules. Increased levels of MG have been reported in the blood of diabetic patients [4]. Protein glycation by MG, unlike that of reducing sugars in mainly arginine-directed, however, other amino acid residues like lysine, histidine and cysteine are also modified [5].

Glycation causes the inactivation of superoxide dismutase (SOD) [6,7]. SOD constitutes an important defence mechanism by which cells counter the deleterious effects of reactive oxygen species (ROS). Cu,Zn-SOD is a homodimeric enzyme, and in bovine erythrocyte SOD, each subunit is folded into eight stranded β-barrel with three major external loops [8]. The damage caused by oxidative stress is expected to be greater if the antioxidant enzymes themselves are inactivated by ROS. Since restriction in the glycation has been shown to alleviate complications associated with diabetes and other related disorders, efforts continue to develop strategies that inhibit the accumulation of ROS.

Black seeds (*Nigella sativa*) (Fig. 2A) have been used extensively in traditional folk medicine [9]. Seeds or their extracts have antidiabetic, antihypertensive, antimicrobial, antiinflammatory, antitumour, galactagogue and insect repellent effects [9–11]. Thymoquinone (TQ) (Fig. 2B) which is the main active component of the volatile oil of black seeds also has antidiabetic activity apart from possessing many other useful pharmacological activities [12–14]. We have recently shown by activity and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis that TQ protects SOD against glycation [15]. The aim of the present

Abbreviations: SOD, superoxide dismutase; TQ, thymoquinone; AGEs, advanced glycation end products; MG, methylglyoxal; ThT, thioflavin T; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; BCA, bicinchoninic acid.

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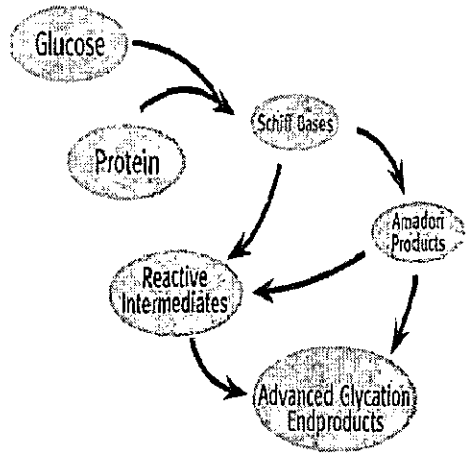

Fig. 1. Stages of protein glycation.

study was to gain insights into the structural changes, formation of AGEs and fibrils that accompany the glycation of SOD by glucose and MG, and its protection by TQ.

2. Materials and methods

2.1. Materials

Bovine erythrocyte Cu,Zn-SOD (EC 1.15.1.1), MG (40% aqueous solution), Thioflavin T (ThT), BSA and bicinchoninic acid (BCA) were purchased from Sigma, USA. Glucose was a product of Qualigens, India. TQ (274666) was obtained from Aldrich. All other chemicals used were of analytical grade.

2.2. SDS–PAGE

SDS–PAGE of SOD was performed using 15 and 5% separating and stacking gels, respectively [16]. Protein bands were visualized by overnight staining with 0.1% (w/v) Coomassie Brilliant Blue R250.

2.3. In vitro glycation of SOD

The concentration of SOD was determined using the BCA method using BSA as the standard [17]. SOD stock (1 mg/ml) was made in 20 mM sodium phosphate buffer, pH 7.4 and stored at −20 °C for future use. To induce glycation, SOD (0.2 mg/ml) was incubated along with 0.5 M glucose, 10 mM MG or a combination of 0.5 M glucose and 10 mM MG in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl. Autoclaved tubes were used to perform the incubations in order to maintain sterile conditions. Bacterial growth was not observed during the periods of incubation. The incubations were carried out in a shaking water bath at 37 °C for 1 h, 1 day, 5 days and 10 days. The native SOD incubated with phosphate buffer (20 mM, pH 7.4 containing 0.15 M NaCl) alone served as the control. Glucose or MG or combination of both glucose and MG-induced glycation of SOD was assessed by absorption, intrinsic fluorescence, AGE-specific fluorescence, ThT fluorescence and far-UV CD spectroscopic studies.

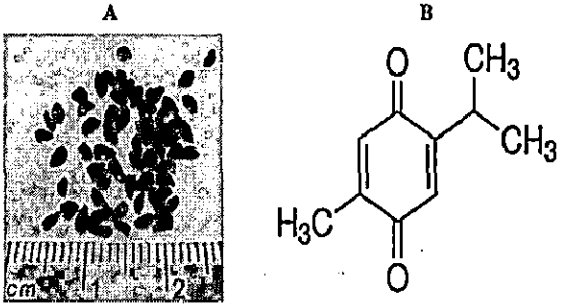

Fig. 2. Seeds of *N. sativa* (A). Chemical structure of thymoquinone (B).

2.4. Effect of TQ on the glycation of SOD

SOD was incubated with 0.5 M glucose or 10 mM MG or combination of 0.5 M glucose and 10 mM MG for 10 days at 37 °C in the presence of 0, 10, 20 and 50 μM TQ. TQ was dissolved in DMSO and the final DMSO concentration in the incubation mixture was 1%. The effect of TQ on the glycation of SOD with glucose or MG or a combination of both were assessed by absorption, intrinsic fluorescence, AGE-specific fluorescence and ThT fluorescence spectroscopic studies. Far-UV CD was not performed in the presence of TQ as DMSO interfered with the measurements in this wavelength range.

2.5. Biophysical studies on the glycation of SOD and its protection by TQ

2.5.1. Absorption spectroscopy

Absorbance measurements were carried out on a double-beam Perkin Elmer spectrophotometer (Lambda 25). The spectra of SOD (0.2 mg/ml) in absence/presence of glucose or MG and absence/presence of TQ were measured in the wavelength range of 240–500 nm.

2.5.2. Fluorescence spectroscopy

All fluorescence measurements were carried out on a Shimadzu spectrofluorometer (model RF-5301PC). The intrinsic fluorescence of SOD (0.2 mg/ml) incubated alone, with glucose or with MG in the absence/presence of TQ was monitored with excitation at 280 nm and emission in the range 290–400 nm. The slit widths were 5 nm for both excitation and emission. The formation of fluorescent AGE products was monitored with excitation at 350 nm and emission in the range 400–480 nm. The slit widths were 3 nm for both excitation and emission. The fibrillar state of incubated SOD was determined via ThT, a reagent used for detecting the β-sheet configuration in proteins [18]. The fluorescence of the above incubation mixtures was monitored after adding 6 μM ThT reagent at excitation wavelength of 440 nm and the emission was measured in the range 450–600 nm. The slit widths were 10 nm for both excitation and emission.

2.5.3. CD-spectroscopy

Far-UV CD was used to measure changes in the secondary structure of SOD (0.2 mg/ml) upon glycation by glucose and MG. The measurements were carried out with a Jasco spectropolarimeter (J-815) equipped with a Jasco Peltier-type temperature controller (PTC-424S/15). The instrument was calibrated with D-10-camphorsulphonic acid. The spectra were collected in a cell of 0.1 cm with scan speed of 100 nm/min and response time of 1 s. Each spectrum was the average of two scans.

3. Results and discussion

3.1. In vitro glycation of SOD

SOD is a dimeric protein of molecular mass of 32 kDa [19]. The enzyme migrated as a single band (16 kDa) in SDS–PAGE and hence was homogenous and, therefore, it was used in the experiments without further purification. Incubation of enzymes with sugars

for long durations has been shown to result in nonenzymatic glycation leading to inactivation [20,21]. MG has been proven to be one of the most reactive glycating agent that can easily react with different groups of amino acids or proteins and form a number of MG derived AGEs [5,22]. In this study, the glycation of SOD was performed with high nonphysiological concentration of glucose (0.5 M) or MG (10 mM) which may serve as an appropriate model for the long-term effects of glucose or MG on the enzyme [22,23]. Glycation-induced structural changes in the enzyme were evaluated by absorption, fluorescence and CD spectroscopy (Fig. 3).

3.1.1. Absorption studies

Bovine erythrocyte Cu,Zn-SOD lacks tryptophan residues and has one tyrosine residue per subunit. SOD that had not been incubated with glucose or MG (native SOD) gave absorbance in the wavelength range 250–300 nm. In glucose, MG or a combination of both glucose and MG-incubated samples, an increasing hyperchromicity was observed with increasing days of incubation. Very slight increase in absorbance at 280 nm with increasing days of incubation at 37 °C was observed in the case of the native SOD (control) (Fig. 3A). The increase in absorbance was much more and faster in the case of SOD incubated with MG than with glucose (Fig. 3A), implying that the structural changes in the enzyme were much more and faster with MG than with glucose. And the increase in absorbance was the most and fastest in the case of SOD incubated with a combination of both glucose and MG and is similar to the effect of MG alone (Fig. 3A) since MG is a stronger glycating agent than glucose, hence the effect of MG dominates over the effect of glucose. The observed hyperchromicity, in the case of glucose-incubated SOD, could be due to modification of aromatic amino acids or changes in the micro environment of aromatic amino acids of the enzyme. The observed hyperchromicity, in the case of MG or glucose + MG, could be due to change in the conformation of SOD due to glycation-induced unfolding leading to cross-linking and aggregation. We have previously reported by SDS–PAGE analysis the formation of cross-linked structures in SOD incubated with MG and not with glucose [15].

3.1.2. Intrinsic fluorescence studies

Total intrinsic fluorescence of native and glycated SOD was measured by exciting at 280 nm. Native SOD gave an emission peak at 310 nm. In glucose, MG or a combination of both glucose and MG glycated samples, an increasing fluorescence quenching was observed with increasing days of incubation, indicating exposure of the aromatic residues to the polar environment [24]. In the control sample, very slight fluorescence quenching at 310 nm was observed with increasing days of incubation (Fig. 3B). Therefore, glycation induced structural changes in SOD incubated with glucose or MG or a combination of both. Fluorescence quenching was much more and faster for MG than for glucose incubated enzyme (Fig. 3B), implying again that MG incubation has a greater effect on the structure of SOD than glucose incubation. And incubation of SOD with both glucose and MG resulted in even more and faster fluorescence quenching than MG alone incubated enzyme (Fig. 3B) implying greatest structural change in this case.

3.1.3. CD studies

Far-UV CD studies in the 200–250 nm wavelength range were performed to measure the changes in the secondary structure [25] of SOD upon glycation. Native SOD gave a negative peak at 208 nm. In glucose, MG or a combination of both glucose and MG glycated samples, a progressive decrease in the negative ellipticity was observed with increasing days of incubation. In the control sample, very slight decrease in negative ellipticity at 208 nm occurred with increasing days of incubation (Fig. 3C). Glycation induced changes in the secondary structure of SOD in all the above three cases. However, the decrease in negative ellipticity was more and faster for MG than for glucose incubated enzyme (Fig. 3C), implying that MG has a greater effect on the secondary structure of SOD than glucose. As expected, the combined effect of both glucose and MG on the glycation of SOD resulted in even more and faster secondary structural changes than MG alone (Fig. 3C).

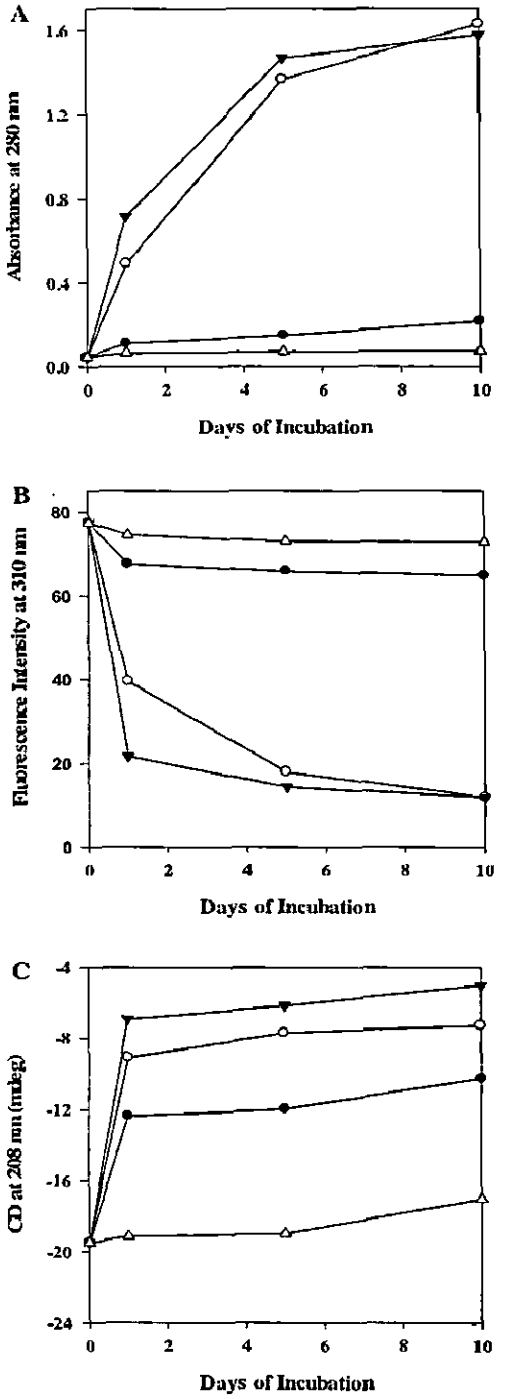

Fig. 3. Effect of glycation of SOD on the absorbance, intrinsic fluorescence and far-UV CD of the enzyme. Absorbance at 280 nm (A), fluorescence intensity at the excitation/emission wavelengths of 280/310 nm (B) and CD at 208 nm (C) for samples of SOD incubated for various days at 37 °C in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (△) and with 0.5 M glucose (●) or 10 mM MG (○) or with a combination of 0.5 M glucose and 10 mM MG (▼).

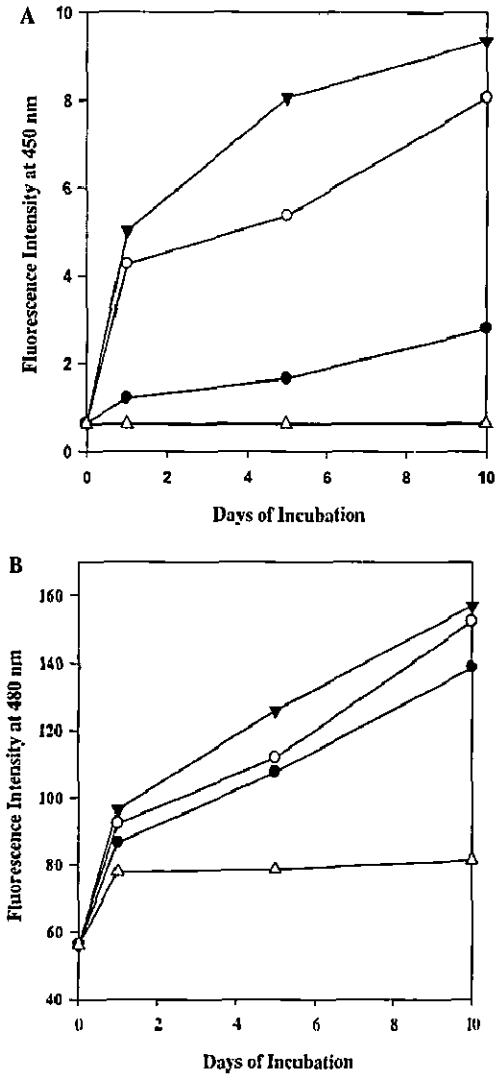

Fig. 4. Effect of glycation of SOD on the AGE-specific and ThT fluorescence of the enzyme. AGE-specific (A) and ThT (B) fluorescence intensity at the excitation/emission wavelengths of 350/450 nm and 440/480 nm, respectively, for samples of SOD incubated for various days at 37 °C in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl alone (△) and with 0.5 M glucose (●) or 10 mM MG (○) or with a combination of 0.5 M glucose and 10 mM MG (▼).

3.1.4. AGE-specific fluorescence studies

We have followed the formation of AGEs in the samples via autofluorescence [18]. Fluorescent AGE-specific fluorescence of native and glycated SOD was measured by exciting at 350 nm. The control sample showed no fluorescence in the wavelength range 400–480 nm. Whereas, SOD incubated with glucose, MG or a combination of both glucose and MG showed AGE-specific fluorescence in the wavelength range 400–480 nm which increased with increasing days of incubation (fluorescence enhancement). The fluorescence enhancement observed at 450 nm with increasing days of incubation in the case of glucose incubated SOD was low, implying few AGEs formed in this case (Fig. 4A) However, the enhancement was much more and faster in the case of MG-incubated enzyme (Fig. 4A), implying formation of larger quantity of AGEs. Incubation of SOD with both glucose and MG resulted in even more and faster fluorescence enhancement than MG alone incubated enzyme (Fig. 4A) implying greatest quantity of AGEs formed in this case. The spectra of fluorescence intensity versus wavelength (400–480 nm) were rather broad (data not shown), and this probably reflects the presence of a number of different fluorescent compounds being formed during glycation.

3.1.5. ThT fluorescence studies

ThT is a dye that interacts with the fibrillar structure of proteins. Upon interaction its fluorescence intensifies, while in its free form is only weakly fluorescent. This quality has been employed in the detection of amyloid fibril structures in proteins [18]. The control sample showed a slight increase in ThT fluorescence at 480 nm with increasing days of incubation at 37 °C (Fig. 4B). Whereas SOD incubated with glucose, MG or a combination of both glucose and MG exhibited much greater fluorescence enhancement (Fig. 4B), and hence enhancement in the fibrillar state, which increased with increasing days of incubation. The fibrillar state enhancement observed in the case of both glucose and MG-incubated SOD was the most, followed by MG-incubated enzyme, and then by glucose-incubated enzyme (Fig. 4B). Therefore, fibrils were formed in all the three cases.

3.2. Protective effect of TQ on the glycation of SOD

3.2.1. Absorption studies

As observed in the earlier experiment (Fig. 3A), glycation of SOD by glucose, MG and both glucose and MG results in hyperchromicity (structural changes). When SOD is incubated for 10 days at 37 °C with glucose, MG or both glucose and MG and increasing concentration of TQ, a progressive decrease in absorbance at 280 nm with increasing TQ concentration was observed in all the three cases (Fig. 5A). SOD incubated alone for 10 days with increasing TQ concentration (control) exhibited no decrease in absorbance at 280 nm (Fig. 5A). Therefore, TQ protected the enzyme to some extent against the structural changes induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of TQ. However, the enzyme was still far from the structure of the native enzyme even at 50 μM concentration of TQ in all the three cases. It was observed that the absorbance at 280 nm for samples of SOD incubated for 10 days with glucose, MG or both and without TQ but 1% DMSO (Fig. 5A) was higher than the same samples incubated without TQ and DMSO (Fig. 3A). Therefore, it appears that even 1% DMSO present in the incubation mixtures decreases the stability of SOD at 37 °C and hence makes it more prone to glycation. It has also been observed in the case of myoglobin and concanavalin A that low concentration of DMSO reduces the thermal stability of both proteins [26].

3.2.2. Intrinsic fluorescence studies

As observed in the earlier experiment (Fig. 3B), glycation of SOD by glucose, MG and both glucose and MG results in intrinsic fluorescence quenching (structural changes). When SOD is incubated for 10 days at 37 °C with glucose, MG or both glucose and MG and increasing concentration of TQ, a progressive increase in fluorescence at 310 nm with increasing TQ concentration was observed in all the three cases (Fig. 5B). The control exhibited insignificant increase in fluorescence at 310 nm (Fig. 5B). Therefore, again this experiment shows that TQ protected the enzyme to some extent against the structural changes induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of TQ. However, it was observed that SOD incubated for 10 days at 37 °C with glucose, without TQ but with 1% DMSO showed fluorescence enhancement at 310 nm and not quenching (Fig. 5B). Therefore, it appears that in the samples of SOD glycated by glucose in the presence of DMSO, the environment around the aromatic residues of the protein is somehow perturbed which affects their fluorescence [27,28].

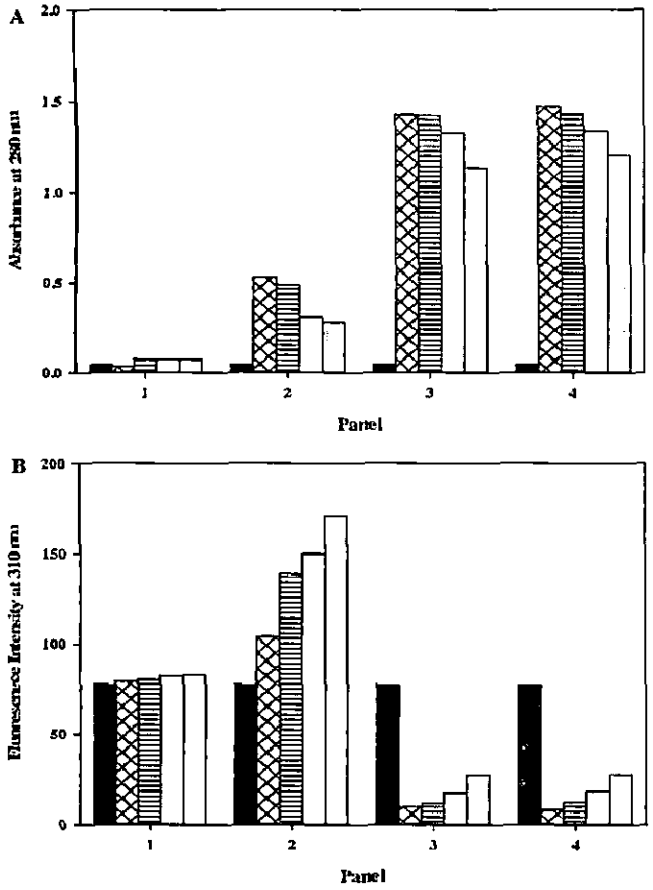

Fig. 5. Effect of TQ on the structural changes induced in SOD due to glycation. Absorbance at 280 nm (A) and fluorescence intensity at the excitation/emission wavelengths of 280/310 nm (B) for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl and 1% DMSO (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37 °C in the presence of 0 (⁂), 10 (=), 20 (□) and 50 (☐) μM TQ. Each panel also shows native SOD alone in buffer that was not incubated at 37 °C for any time period (■).

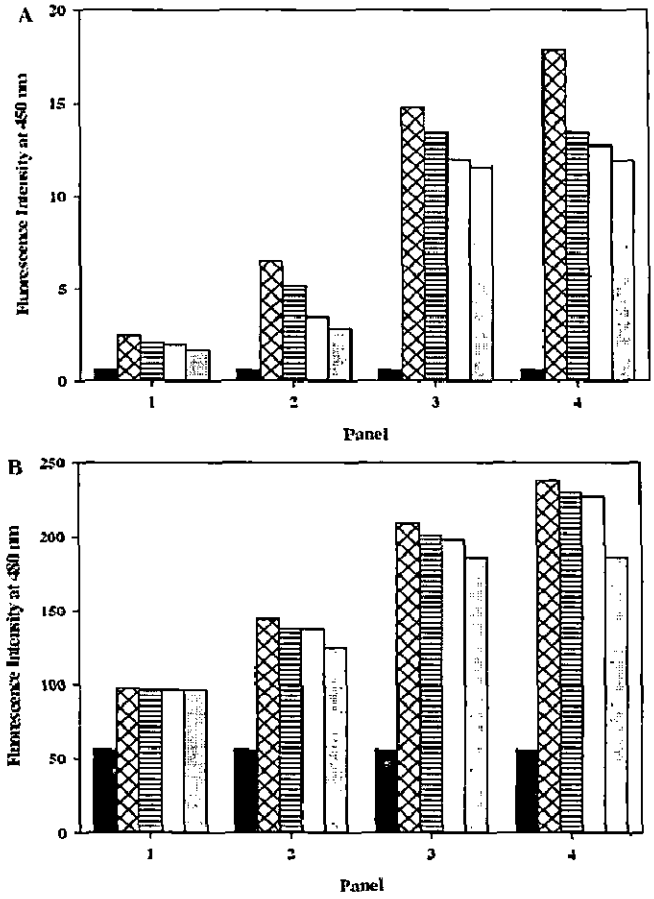

Fig. 6. Effect of TQ on the fluorescent AGEs and fibrils formed in SOD due to glycation. AGE-specific (A) and ThT (B) fluorescence intensity at the excitation/emission wavelengths of 350/450 nm and 440/480 nm, respectively, for samples of SOD (0.2 mg/ml) that were incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl and 1% DMSO (Panel 1) and with 0.5 M glucose (Panel 2) or 10 mM MG (Panel 3) or a combination of 0.5 M glucose and 10 mM MG (Panel 4) for 10 days at 37 °C in the presence of 0 (⁂), 10 (=), 20 (□) and 50 (☐) μM TQ. Each panel also shows native SOD alone in buffer that was not incubated at 37 °C for any time period (■).

3.2.3. *AGE fluorescence studies*

As observed in the earlier experiment (Fig. 4A), glycation of SOD by glucose, MG and both glucose and MG results in AGE-specific fluorescence (formation of AGEs). When SOD is incubated for 10 days at 37 °C with glucose, MG or both glucose and MG and increasing concentration of TQ, a progressive decrease in AGE-specific fluorescence at 450 nm with increasing TQ concentration was observed in all the three cases (Fig. 6A). The control exhibited insignificant decrease in fluorescence at 450 nm (Fig. 6A). Therefore, TQ protected the enzyme to some extent against formation of AGEs induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of TQ. It was again observed that the presence of 1% DMSO in the incubation mixtures decreases the stability of SOD at 37 °C and hence makes it more prone to glycation and subsequent AGEs formation.

3.2.4. *ThT fluorescence studies*

As observed in the earlier experiment (Fig. 4B), glycation of SOD by glucose, MG and both glucose and MG results in ThT fluorescence enhancement (formation of fibrils). When SOD is incubated for 10 days at 37 °C with glucose, MG or both glucose and MG and increasing concentration of TQ, a progressive decrease in ThT fluorescence at 480 nm with increasing TQ concentration was observed in all the three cases (Fig. 6B). The control exhibited insignificant decrease in ThT fluorescence at 480 nm (Fig. 6B). Therefore, TQ protected the enzyme to some extent against formation of fibrils induced by glycation with glucose, MG or a combination of both, and the protection increased with increasing concentration of TQ. It was again observed that the presence of 1% DMSO in the incubation mixtures decreases the stability of SOD at 37 °C and hence makes it more prone to glycation and subsequent fibril formation.

4. Conclusion

TQ offered protection against glucose or MG-induced glycation of SOD as observed by a reduction in the structural changes, formation of AGEs and fibrils. This structural study along with our recent report [15] firmly establish TQ as an antiglycating agent, and this along with its antidiabetic effects makes it an effective compound in treating diabetes and its complications.

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